Exhibit 1

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
61/565,358	11/30/2011		125	75820.210001		

CONFIRMATION NO. 1601

FILING RECEIPT

21967
HUNTON & WILLIAMS LLP
INTELLECTUAL PROPERTY DEPARTMENT
2200 Pennsylvania Avenue, N.W.
WASHINGTON, DC 20037

OC00000051799429

Date Mailed: 01/06/2012

Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Erin A. Kimbrel, Worcester, MA;

Power of Attorney:
Alexander Spiegler--56625

If Required, Foreign Filing License Granted: 01/04/2012

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 61/565,358**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No
** **SMALL ENTITY** **
Title

METHODS OF GENERATING MESENCHYMAL STROMAL CELLS USING HEMANGIOBLASTS

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international

patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and

Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

PTO/SB/16 (10-07) modified)

Attorney Docket Number:	75820.210001

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

THIS IS A REQUEST FOR FILING A PROVISIONAL APPLICATION FOR PATENT UNDER 37 C.F.R. § 1.53(C).

INVENTOR(S)

Given Name (first and middle (if any))	Family Name or Surname	Residence (City and Either State or Foreign Country)
Erin A.	**Kimbrel**	**Worcester, MA**

☐ Additional inventors are being named on page 2 attached hereto.

TITLE OF THE INVENTION (500 characters max):

METHODS OF GENERATING MESENCHYMAL STROMAL CELLS USING HEMANGIOBLASTS

CORRESPONDENCE ADDRESS

Direct all correspondence to:

☒ The address corresponding to Customer Number: **21967**

OR

☐ Firm Name	**Hunton & Williams LLP**				
Address	**Intellectual Property Department**				
	2200 Pennsylvania Avenue, N.W.				
City	**Washington**	State	**DC**	Zip Code	**20037**
Country	**U.S.A.**	Telephone	**202-955-1500**	Facsimile	**202-778-2201**

ENCLOSED APPLICATION PARTS (check all that apply)

☐	Application Data Sheet. See 37 CFR 1.76	☐	CD(s). Number of CDs _____
☒	Specification Number of Pages **51**	☐	Other (specify) _____
☒	Drawing(s) Number of Sheets **14**		

Fees Due: - Filing Fee of $220 ($110 for small entity). If the specification and drawings exceed 100 sheets of paper, an application size fee is also due, which is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s)

Total Sheets	Extra Sheets	No. of Each Additional 50 or Fraction Thereof	Fee Due ($)	Fee Paid ($)
_____ - 100 =	_____ / 50 =	_____ (round up to a whole number) x	_____ =	_____

METHOD OF PAYMENT OF THE FILING FEE AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

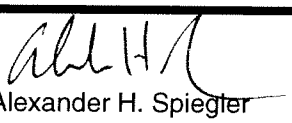
☐ A check in the amount of ☐ $_____ is enclosed to cover the filing fee and application size fee (if applicable). The Director is hereby authorized to charge any variance between the amount enclosed and the Patent Office charges to **Deposit Account No. 50-0206**.

☒ The Director is hereby authorized to charge the filing fee and application size fee (if applicable) or credit any overpayment to Deposit Account No. **50-0206**.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Signature		Date	November 30, 2011
Typed or Printed Name	Alexander H. Spiegler	Registration No.	56,625
Telephone No.	202-955-1500		

METHODS OF GENERATING MESENCHYMAL STROMAL CELLS USING HEMANGIOBLASTS

Background

(a) Field

[001] The present disclosure generally relates to methods of generating mesenchymal stromal cells from hemangioblasts. These methods produce substantial numbers of high quality mesenchymal stromal cells (MSCs). The resulting mesenchymal stromal cells are useful in the treatment of various diseases and conditions such as multiple sclerosis and other autoimmune disorders.

(b) Description of the Related Art

[002] Multiple sclerosis (MS) is an autoimmune disorder caused by inflammation of the myelin sheath surrounding the neuronal axons in the central nervous system (CNS). MS patients suffer from a variety of neurologic symptoms including paralysis. There are about 400,000 MS patients in the U.S. alone, yet currently there is no cure or efficient therapies for this disease. It has been found that bone marrow-derived mesenchymal stromal cells (BM-MSCs) [1-5] or adipose-derived MSCs [6] can prevent or delay the onset of experimental autoimmune encephalomyelitis (EAE) — an animal model for MS and other demyelinating diseases of the CNS. However, the limited sources and varying quality of bone marrow or fat donors restrict the study and application of MSCs from these sources. Therefore, scientists have sought to use human embryonic stem cells (hESCs) as another source for MSCs (i.e., creating MSCs derived from hESCs or hESC-MSCs).

[003] hESC-MSCs can be generated in a long-term supply and highly controllable manner, thus alleviating the problems with donor-dependent sources. Since long-term engraftment of

MSCs is not required, there is basically no concern for mismatch of major histocompatibility (MHC) [7, 8]. hESC-MSCs have been derived through various methods including co-culture with murine OP9 cells or handpicking procedures [9-13]. These methods, however, are tedious and generate hESC-MSCs with a low yield and potentially varying quality.

Summary of Preferred Embodiments

[004] The present disclosure relates to methods for generating mesenchymal stromal cells (MSCs) by culturing hemangioblasts (e.g., hemangioblasts differentiated from hESCs), MSCs derived from hemangioblasts, and methods of using such hemangioblast-derived MSCs. As compared to conventional methods for deriving hESC-MSCs described above, embodiments of the subject methods can produce MSCs more quickly, with less labor, with higher yield and greater purity in the resulting cells, and with a decrease in quality variation. In particular, the hemangioblast-derived mesenchymal stromal cells described herein (a) do not clump or clump substantially less than mesenchymal stromal cells derived directly from hESCs; (b) more easily disperse when splitting compared to mesenchymal stromal cells derived directly from hESCs; (c) are greater in number than mesenchymal stromal cells derived directly from hESCs when starting with equivalent numbers of hESCs; and/or (d) acquire characteristic mesenchymal cell surface markers faster than mesenchymal stromal cells derived directly from hESCs.

[005] The present disclosure relates to methods for generating mesenchymal stromal cells (e.g., human MSCs) involving the culture of hemangioblasts in, for example, feeder-free conditions and plated on a matrix. In one embodiment, the matrix comprises transforming growth factor beta (TGF-beta), epidermal growth factor (EGF), insulin-like growth factor 1, bovine fibroblast growth factor (bFGF), and/or platelet-derived growth factor (PDGF), laminin, fibronectin, vitronectin, proteoglycan, entactin, collagen, collagen I, collagen IV, heparan sulfate,

Matrigel (a soluble preparation from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells), a human basement membrane extract, or any combination thereof. In another embodiment, the matrix is from human or non-human animal (e.g., bovine, mouse or rat origin).

[006] In another embodiment, the hemangioblasts are cultured in a medium comprising serum or a serum replacement such as αMEM supplemented with 20% fetal calf serum. In another embodiment, the hemangioblasts are cultured on the matrix for at least 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 days. In a particular embodiment, the hemangioblasts are cultured on Matrigel for about 7 days and then transferred off Matrigel to grow on uncoated tissue culture dish for an additional 14-23 days (or more to increase yield of the resulting MSCs).

[007] In other embodiments, the hemangioblasts are differentiated from ESCs such as iPS, MA09, H7, H9, MA01, HuES3, or H1gfp cells. In another embodiment, the ESCs are derived from one or more inner cell mass cells or one or more blastomeres.

[008] In another embodiment, the hemangioblasts are differentiated from ESCs by

(a) culturing ESCs in, for example, the presence of vascular endothelial growth factor (VEGF) and/or bone morphogenic protein 4 (BMP-4) to form embryoid bodies;

(b) culturing said embryoid bodies in the presence of at least one growth factor (e.g., basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and bone morphogenic protein 4 (BMP-4), stem cell factor (SCF), Flt 3L (FL), thrombopoietin (TPO), and/or tPTD-HOXB4) in an amount sufficient to induce the differentiation of said embryoid bodies into hemangioblasts; and

(c) culturing said hemangioblasts in a medium comprising at least one additional growth factor (e.g., insulin, transferrin, granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF),

erythropoietin (EPO), stem cell factor (SCF), vascular endothelial growth factor (VEGF), bone morphogenic protein 4 (BMP-4), and/or tPTD-HOXB4), wherein said at least one additional growth factor is in an amount sufficient to expand human hemangio-colony forming cells in said culture.

[009] In another embodiment, the hemangioblasts are harvested after at least 6, 7, 8, 9, 10, 11, 12, 13, or 14 days of starting to induce differentiation of ESCs. In another embodiment, mesenchymal stromal cells are generated within at least 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 days of starting to induce differentiation of said ESCs.

[010] In another embodiment, at least 80, 85, 90, 95, 100, 125, or 150 million mesenchymal stromal cells are generated from, for example, about 200,000 hemangioblasts within, for example, at least 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 days of culturing the hemangioblasts. In another embodiment, the mesenchymal stromal cells comprise less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%, 0.009%, 0.008%, 0.007%, 0.006%, 0.005%, 0.004%, 0.003%, 0.002%, 0.001%, 0.0009%, 0.0008%, 0.0007%, 0.0006%, 0.0005%, 0.0004%, 0.0003%, 0.0002%, or 0.0001% human embryonic stem cells. In another embodiment, the mesenchymal stromal cells are substantially purified with respect to human embryonic stem cells and comprise at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% mesenchymal stromal cells. In another embodiment, the mesenchymal stromal cells do not form teratomas when introduced into a host animal (e.g., an immunocompromised host animal). In another embodiment, at least 50% of the mesenchymal stromal cells are positive for CD105 or CD73 after 15 days of culture. In another embodiment, at least 80% of the

mesenchymal stromal cells are positive for CD105 and CD73 within 20 and 21 days of culture, respectively. In another embodiment, the mesenchymal stromal cells are capable of undergoing at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, or more population doublings in culture.

[011] In another embodiment, the disclosure provides a kit comprising the mesenchymal stromal cells described herein. In another embodiment, the disclosure provides a pharmaceutical preparation comprising the mesenchymal stromal cells described herein.

[012] In another embodiment, the disclosure provides for a method of treating a disease or disorder by administering an effective amount of mesenchymal stromal cells derived from hemangioblasts to a subject in need thereof. The disease or disorder may include, but is not limited to autoimmune disorder, uveitis, bone loss or cartilage damage. mesenchymal stromal In another embodiment, the mesenchymal stromal cells are administered in combination with an allogeneic transplanted cell or tissue such as a retinal pigment epithelium cell, retinal cell, corneal cell, or muscle cell.

Brief Description of the Drawings

[013] FIG. 1 shows a microscopic view of generating MSCs from hESCs via hemangioblasts.

[014] FIG. 2 shows the percentage of cells positive for MSC surface markers in the initial hemangioblast population (left side of graph, day 7-11 hemangioblast) and after culturing hemangioblasts on Matrigel coated plates (right side of graph) and a microscopic view of the MSCs derived from the hemangioblasts (right panel photograph).

[015] FIG. 3 shows the percentage of cells positive for MSC surface markers after culturing human embryonic stem cells (hESC) on gelatin coated plates (left panel), hESC on Matrigel coated plates (middle panel), and hemangioblasts on Matrigel coated plates (right panel).

[016] FIG. 4 shows the yields of cells positive for MSC surface markers obtained from culturing hESC on gelatin coated plates (first column - no yield), hESC on Matrigel coated plates (second column), and hemangioblasts on Matrigel coated plates (third column).

[017] FIG. 5 depicts the time for MSC surface markers to be acquired using hemangioblasts (top line) and hESC (lower line).

[018] FIG. 6 shows the percentage of cells positive for MSC markers and negative for hematopoiesis and endothelial markers after culturing hESC on Matrigel coated plates (left panel) and hemangioblasts on Matrigel coated plates (right panel).

[019] FIG. 7 depicts the differentiation capabilities of MSCs derived from hemangioblasts differentiated from MA09 hESC to form adipocytes and osteocytes.

[020] FIG. 8 depicts chondrogenic differentiation of MA09 hESC hemangioblast-derived MSCs by mRNA expression of Aggrecan (chondroitin proteoglycan sulfate 1) and Collagen IIa.

[021] FIG. 9 shows the results of a pilot animal study to treat experimental autoimmune encephalomyelitis (EAE) with MSCs derived from hemangioblasts versus vehicle control.

[022] FIG. 10 shows the transient expression of the cell surface marker CD309.

[023] FIG. 11a shows hemangioblast-derived MSCs suppression of T cell proliferation caused by chemical stimulation (PMA/ionomycin).

[024] FIG. 11b shows hemangioblast-derived MSCs suppression of T cell proliferation caused by exposure to dendritic cells.

[025] FIG. 12a shows that hemangioblast-derived MSCs were able to increase the percentage of CD4/CD25 double positive Tregs that are induced in response to IL2 stimulus.

[026] FIG. 12b shows that hemangioblast-derived MSCs inhibit Th1 secretion of IFNγ.

[027] FIG. 13 shows that interferon gamma stimulates changes in MSC surface marker expression and may enhance MSC immunosuppressive effects.

Detailed Description of Preferred Embodiments

[028] The present disclosure relates to methods of generating mesenchymal stromal cells (MSCs) by culturing hemangioblasts, MSCs obtained by these methods, and methods of treatment using these MSCs.

[029] The inventor has surprisingly found that the hemangioblast-based methods produce increased yields of hESC-derived MSCs compared to prior methods. Moreover, the hemangioblast-based methods produce hESC-derived MSCs more efficiently than previous methods and greatly reduces the concern of contamination of residual hESCs in the final MSC population. Accordingly, the inventors have discovered that, going through an intermediate hemangioblast stage prior to further differentiation, permits a rapid expansion of multipotent cells, facilitates large scale production of mature cell populations further downstream, and does not require labor-intensive hand-picking.

Definitions

[030] "Pluripotent cells," as used herein, refers broadly to a cell capable of prolonged or virtually indefinite proliferation *in vitro* while retaining their undifferentiated state, exhibiting a stable (preferably normal) karyotype, and having the capacity to differentiate into all three germ layers (*i.e.*, ectoderm, mesoderm and endoderm) under the appropriate conditions. Typically pluripotent cells (a) are capable of inducing teratomas when transplanted in immunodeficient (SCID) mice; (b) are capable of differentiating to cell types of all three germ layers (e.g., ectodermal, mesodermal, and endodermal cell types); and (c) express at least one hES cell

marker (such as Oct-4, alkaline phosphatase, SSEA 3 surface antigen, SSEA 4 surface antigen, NANOG, TRA 1 60, TRA 1 81, SOX2, REX1). Exemplary pluripotent cells include but are not limited to ES cells, iPS cells, pluripotent cells produced from embryonic germ (EG) cells (e.g., by culturing in the presence of FGF-2, LIF and SCF), parthenogenetic ES cells, ES cells produced from cultured inner cell mass cells, ES cells produced from a blastomere, and ES cells produced by nuclear transfer (e.g., a somatic cell nucleus transferred into a recipient oocyte). Exemplary pluripotent cells may be produced without destruction of an embryo. For example, iPS cells may be produced from cells obtained without embryo destruction. As a further example, ES cells may be produced from a biopsied blastomere (which can be accomplished without harm to the remaining embryo); optionally, the remaining embryo may be cryopreserved, cultured, and/or implanted into a suitable host.

[031] "Embryo" or "embryonic," as used herein refers broadly to a developing cell mass that has not implanted into the uterine membrane of a maternal host. An "embryonic cell" is a cell isolated from or contained in an embryo. This also includes blastomeres, obtained as early as the two-cell stage, and aggregated blastomeres.

[032] "Embryonic stem cells" (ES cells or ESC), as used herein, refers broadly to pluripotent cells derived from an embryo (e.g., from the inner cell mass of blastocysts or one or more blastomeres (optionally without destroying the remainder of the embryo)) that have been serially passaged as cell lines. Many methods of generating ES cells are known in the art. ES cells may be derived from an embryo produced by any method, such as fertilization of an egg cell with sperm or sperm DNA, nuclear transfer, or parthenogenesis. For example, embryonic stem cells also include cells produced by somatic cell nuclear transfer, even when non-embryonic cells are used in the process. For example, ES cells may be derived from the ICM of blastocyst stage

embryos, as well as embryonic stem cells derived from one or more blastomeres Such embryonic stem cells can be generated from embryonic material produced by fertilization or by asexual means, including somatic cell nuclear transfer (SCNT), parthenogenesis, and androgenesis. Further exemplary pluripotent stem cells include induced pluripotent stem cells (iPS cells) generated by reprogramming a somatic cell by contacting the cell with one or more reprogramming factors. For example, the reprogramming factor(s) may be expressed by the cell, e.g., from an exogenous nucleic acid added to the cell, or from an endogenous gene in response to a factor such as a small molecule, microRNA, or the like that promotes or induces expression of that gene (see Suh and Blelloch, Development 138, 1653-1661 (2011); Miyosh et al., Cell Stem Cell (2011), doi:10.1016/j.stem.2011.05.001; Sancho-Martinez et al., Journal of Molecular Cell Biology (2011) 1–3; Anokye-Danso et al., Cell Stem Cell 8, 376–388, April 8, 2011; Orkin and Hochedlinger, Cell 145, 835-850, June 10, 2011, each of which is incorporated by reference herein in its entirety). Reprogramming factors may be provided from an exogenous source, e.g., by being added to the culture media, and may be introduced into cells by methods known in the art such as through coupling to cell entry peptides, protein or nucleic acid transfection agents, lipofection, electroporation, biolistic particle delivery system (gene gun), microinjection, and the like. iPS cells can be generated using fetal, postnatal, newborn, juvenile, or adult somatic cells. In certain embodiments, factors that can be used to reprogram somatic cells to pluripotent stem cells include, for example, a combination of Oct4 (sometimes referred to as Oct 3/4), Sox2, c-Myc, and Klf4. In other embodiments, factors that can be used to reprogram somatic cells to pluripotent stem cells include, for example, a combination of Oct-4, Sox2, Nanog, and Lin28. In other embodiments, somatic cells are reprogrammed by expressing at least 2 reprogramming factors, at least three reprogramming factors, or four reprogramming factors. In other

embodiments, additional reprogramming factors are identified and used alone or in combination with one or more known reprogramming factors to reprogram a somatic cell to a pluripotent stem cell. iPS cells typically can be identified by expression of the same markers as embryonic stem cells, though a particular iPS cell line may vary in its expression profile. ES cells may be generated with homozygosity or hemizygosity in one or more HLA genes, e.g., through genetic manipulation, screening for spontaneous loss of heterozygosity, etc. Embryonic stem cells, regardless of their source or the particular method used to produce them, typically possess one or more of the following attributes: (i) the ability to differentiate into cells of all three germ layers, (ii) expression of at least Oct-4 and alkaline phosphatase, and (iii) the ability to produce teratomas when transplanted into immunocompromised animals. Embryonic stem cells that may be used in embodiments of the present invention include, but are not limited to, human ES cells ("hESC" or "hES cells") such as MA01, MA09, ACT-4, No. 3, H1, H7, H9, H14 and ACT30 embryonic stem cells. The human ES cells used in accordance with exemplary embodiments of the present invention may be derived and maintained in accordance with GMP standards.

[033] Exemplary hES cell markers include but are not limited to: such as alkaline phosphatase, Oct-4, Nanog, Stage-specific embryonic antigen-3 (SSEA-3), Stage-specific embryonic antigen-4 (SSEA-4), TRA-1-60, TRA-1-81, TRA-2-49/6E, Sox2, growth and differentiation factor 3 (GDF3), reduced expression 1 (REX1), fibroblast growth factor 4 (FGF4), embryonic cell-specific gene 1 (ESG1), developmental pluripotency-associated 2 (DPPA2), DPPA4, telomerase reverse transcriptase (hTERT), SALL4, E-CADHERIN, Cluster designation 30 (CD30), Cripto (TDGF-1), GCTM-2, Genesis, Germ cell nuclear factor, and Stem cell factor (SCF or c-Kit ligand).

Hemangioblasts

[034] Hemangioblasts (blasts) are multipotent and serve as the common precursor to both hematopoietic and endothelial cell lineages. During embryonic development, they are believed to arise as a transitional cell type that emerges during early mesoderm development and colonizes primitive blood islands (Choi et al. Development 125 (4): 725-732 (1998). Once there, hemangioblasts are capable of giving rise to both primitive and definitive hematopoietic cells, HSCs, and endothelial cells (Mikkola et al, J. Hematother. Stem Cell Res 11(1): 9-17 (2002).

[035] Hemangioblasts may be derived *in vitro* from both mouse ESCs (Kennedy et al, Nature (386): 488-493 (1997); Perlingeiro et al, Stem Cells (21): 272-280 (2003)) and human ESCs (ref. 14, 15, Yu et al., Blood 2010 116: 4786-4794). Other studies claim to have isolated hemangioblasts from umbilical cord blood (Bordoni et al, Hepatology 45 (5) 1218-1228), circulating CD34- lin- CD45- CD133- cells from peripheral blood (Ciraci et al, Blood 118: 2105-2115), and from mouse uterus (Sun et al, Blood 116 (16): 2932-2941 (2010)). Both mouse and human ESC-derived hemangioblasts have been obtained through the culture and differentiation of embryoid bodies grown in liquid culture followed by growth of the cells in semi-solid medium containing various cytokines and growth factors (Kennedy, Perlingeiro, ref 14, 15); *see also*, U.S. Patent No. 8,017,393, which is hereby incorporated by reference in its entirety. Hemangioblasts useful in the methods described herein may be derived or obtained from any of these known methods.

[036] For example, in one embodiment, a hESC line is cultured to form embryoid bodies. The embryoid bodies (e.g., 3-4 days old) are then induced to differentiate into hemangioblasts through the use of a cytokine-rich serum-free methylcellulose based medium (14, 15). FIG. 1 depicts such an embodiment.

[037] Exemplary hESC lines that may be used to obtain hemangioblasts include, but are not limited to, MA09, H7, H9, MA01, HuES3, and H1gfp.

[038] In other embodiments, hemangioblasts may be derived from any pluripotent cell as described herein. For example, ESCs derived from or using blastocysts, plated ICMs, one or more blastomeres, or other portions of a pre-implantation-stage embryo or embryo-like structure, regardless of whether produced by fertilization, somatic cell nuclear transfer (SCNT), parthenogenesis, androgenesis, or other sexual or asexual means, or ESC derived through reprogramming (e.g., iPS cells) may be used. In a particular embodiment, hemangioblasts may be obtained from hESC such as iPS cells. The iPS cell may be produced using exogenously added factors or other methods known in the art such as proteins or microRNA (*see* Zhou et al., Cell Stem Cell (4): 1-4, 2009; Miyoshi et al. Cell Stem Cell (8): 1-6, 2011; Danso et al., Cell Stem Cell (8): 376-388, 2011).

Methods of Generating MSCs

[039] The present disclosure relates to methods of generating MSCs using hemangioblasts. In one embodiment, hemangioblasts are harvested after at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 days, preferably 6-14 days, of being cultured in, for example, serum-free methylcellulose plus cytokines as described below. The medium may, for example, comprise a base medium methycellulose product H4536 (StemCell Technologies) plus penicillin/streptomycin (pen/strp), Excyte growth supplement (Millipore), and the cytokines, Flt3-ligand (FL) at 50ng/ml, vascular endothelial growth factor (VEGF) at 50ng/ml, thrombopoietin (TPO) at 50ng/ml, and basic fibroblast growth factor (bFGF) at 20 ng/ml. Alternatively, the medium may comprise a base medium methylcellulose product H4236 (Stem Cell Technologies) plus 20% Iscove's Modified Dulbecco's Media (IMDM), pen/strep, Excyte,

50 ng/ml stem cell derived factor (SCF), 20 ng/ml granulocyte macrophage colony stimulating factor (GM-CSF), 20 ng/ml interleukin 3 (IL3), 20ng/ml interleukin 6 (IL6), 50 ng/ml FL, 50 ng/ml VEGF, 50 ng/ml TPO, and 30 ng/ml bFGF. The hemangioblasts are then re-plated and cultured for at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, or 36 days, preferably 14-30 days, to form MSCs. It will be understood that various ranges within these culture periods are envisioned (e.g., hemangioblasts harvested after 8-12 days of culture, re-plated and cultured for 14-30 days), as well as combinations of these various ranges.

[040] In one embodiment, the hemangioblasts are harvested, replated and cultured in liquid medium under feeder-free conditions (i.e., no feeder layer of cells such as mouse embryonic fibroblasts, OP9 cells, etc.). In a particular embodiment, hemangioblasts are cultured on an extracellular matrix, such as a matrix comprising Matrigel (a soluble preparation from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells that gels at room temperature to form a reconstituted basement membrane). More particularly, hemangioblasts are cultured on Matrigel for at least 7 days prior to being transferred to non-coated tissue culture plate (e.g., for 14-23 days). Hemangioblasts may also be cultured on a substrate comprising one or more of the factors that has been reported to be present in Matrigel, including transforming growth factor beta (TGF-beta), epidermal growth factor (EGF), insulin-like growth factor 1, bovine fibroblast growth factor (bFGF), and/or platelet-derived growth factor (PDGF). Hemangioblasts may also be cultured on a matrix comprising Human Basement Membrane Extract (BME) (e.g., Cultrex BME, Trevigen) or an EHS matrix. Additional exemplary matrix components and component mixtures on which hemangioblasts may be cultured include laminin, fibronectin, vitronectin, proteoglycan, entactin, collagen (e.g., collagen I, collagen IV), heparan sulfate, and the like,

alone or in various combinations, e.g., fibronectin/laminin. Said matrix or matrix components may be of human or non-human animal origin (such as bovine, mouse or rat origin). For example, the matrix, matrix component, or combination may be suited on a plastic or other substrate. In a more particular embodiment, hemangioblasts are cultured in a liquid medium comprising serum (e.g., fetal calf serum or human AB serum) on the Matrigel-coated plate. For example, the culture medium may comprise, consist essentially of, or consist of αMEM (Sigma-Aldrich) supplemented with 10-20% fetal calf serum (αMEM+20% FCS), αMEM supplemented with 10-20% heat-inactivated human AB serum, or, IMDM supplemented with 10-20% heat inactivated AB human serum.

[041] In another embodiment, MSC surface markers are acquired faster in hemangioblast-derived MSCs compared to hESC-derived MSCs. *See* FIG. 5. The acquisition of individual markers may vary depending on the specific hESC line used, the passage number of the hESCs, and/or the number of days grown as hemangioblasts. For example, in one embodiment, the invention provides that at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or 100% of MSCs derived from hemangioblasts are positive for MSC markers (e.g., CD105, CD73) after 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 days in culture. *See* Figure 5. For example, in one embodiment, at least 50% of MSCs derived from hemangioblasts are positive for CD105 and CD73 after 15 days of culture. In another embodiment, at least 80% MSCs derived from hemangioblasts are positive for CD105 and CD73 after 20 and 21 days of culture, respectively. It will be understood that additional embodiments are contemplated within Figure 5. For example, the invention contemplates comparisons of MSCs derived from hemangioblasts to MSCs derived directly from hESCs (e.g., 50% of MSCs

derived from hemangioblasts expressed CD105 at least 7 days faster than 50% of MSCs derived directly from hESCs expressed CD105).

MSCs Derived From Hemangioblasts

[042] The disclosure also relates to the number of MSCs derived from hemangioblasts. Using the methods described herein, substantial numbers of MSCs may be obtained. For example, the disclosure provides for at least 8 x 10^7, 8.5 x 10^7, 9 x 10^7, 9.5 x 10^7, 1 x 10^8, 1.25 x 10^8, or 1.5 x 10^8 MSCs derived from, for example, 2 x 10^5 hemangioblasts within, for example, 30 days of culture as MSCs. Continued expansion of the resulting MSCs will considerably increase this yield and depend upon the exact number of additional days used for culture and expansion. In an alternative embodiment, MSCs may be generated from hemangioblasts in a ratio of hemangioblasts to MSCs of at least 1:400, 1:415, 1:425, 1:440; 1:450, 1:465, 1:475, 1:490, and 1:500, within, for example, 30 days of culture as MSCs. These numbers of MSCs may be obtained within at least 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, or 48 days of culture total, going from the undifferentiated hESC state through the embryoid body stage (3-4 days), hemangioblast stage (6-14 days), and finally through the MSC differentiation stage (14-30).

[043] The exact number of MSCs that will be generated depends on many factors, including but not limited to, the starting number of hESCs, the passage number and general health of the hESCs, the exact media used at each step of differentiation, the number of days cultured as EBs, days cultured in hemangioblast growth medium, and finally number of days cultured under MSC conditions. Nonetheless, in exemplary embodiments of the invention as described herein, the number of MSCs obtained via hemangioblasts is significantly (e.g., at least 5 times, 10 times, 20 times, 22 times) higher than the number of MSCs obtained directly from hESCs. *See* Figure 4.

[044] In another embodiment, the MSCs are substantially free of hESCs or other identifiable cell types after, for example, 30 days of being grown in MSC culture conditions or consist of MSCs. For example, the MSCs may comprise less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%, 0.009%, 0.008%, 0.007%, 0.006%, 0.005%, 0.004%, 0.003%, 0.002%, 0.001%, 0.0009%, 0.0008%, 0.0007%, 0.0006%, 0.0005%, 0.0004%, 0.0003%, 0.0002%, or 0.0001% non-MSC cell types (e.g., hESCs), and preferably contain less than 5% non-MSC cell types. In another embodiment, the MSCs are substantially purified, with respect to non-MSCs (e.g., hESCs), comprising at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% MSCs, and preferably at least 95% MSCs. Methods of sensitive detection of particular cell type (e.g., hESCs) within a larger population of cells of other types (e.g., MSCs) are known in the art. *See* PCT/US11/45232, which is hereby incorporated by reference in its entirety. In a preferred embodiment, the MSCs do not contain any hESCs. In another embodiment, the MSCs do not form teratomas, e.g., when introduced into an immunocompromised animal or a human host (e.g., an immune-compatible host, or when introduced into an immunologically privileged site in a human or non-human host.

[045] In another embodiment, the hemangioblast-derived MSCs possess phenotypes of younger cells as compared to adult-derived MSCs. In one embodiment, the MSCs are capable of undergoing at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, or more population doublings in culture. Adult-derived cells typically undergo 2-3 doublings in culture. In another embodiment, the hemangioblast-derived MSCs have longer telomere lengths, greater immunosuppressive effects, fewer vacuoles, divide faster, divide more readily in culture, higher CD90 expression, less lineage committed, or combinations thereof, compared to adult-derived

MSCs. In another embodiment, the hemangioblast-derived MSC have increased expression of transcripts promoting cell proliferation (i.e., have a higher proliferative capacity) and reduced expression of transcripts involved in terminal cell differentiation compared to adult-derived MSCs.

[046] In another embodiment, the hemangioblast-derived MSCs have improved characteristics compared to MSCs derived directly from hESCs. For example, hESC derived MSCs clump more, are more difficult to disperse when splitting, do not generate nearly as many MSCs when starting with equivalent numbers of hESCs, and take longer to acquire characteristics MSC cell surface markers compared to hemangioblast-derived MSCs. *See* Example 2 and Figures 3-6.

[047] In another embodiment, the hemangioblast-derived MSCs have particular immunosuppressive activity (e.g., cytokine release per MSC or stimulating an increase in the number of regulatoryT cells per MSC, or inhibiting a certain amount of IFN gamma release from Th1 cells per MSC, or stimulating a certain amount of IL4 secretion from Th2 cells per MSC). For example, in one embodiment, the cells potency may be tested, e.g., by a non-specific potency assay measuring cytokine release following a certain stimulation. Cytokines specifically released from MSCs may include but are not limited to transforming growth factor beta, indoleamine 2, 3dioxygenase, prostaglandin E2, hepatocyte growth factor, nitric oxide, interleukin 10, interleukin 6, macrophage-colony stimulating factor, and soluble human leukocyte antigen (HLA) G5. The cells may be considered potent if, for example, HLA G5 release is greater than about 50 pg/mL, greater than about 100 pg/mL, greater than about 150 pg/mL, or greater than about 200 pg/mL.

Pharmaceutical Preparations of MSCs

[048] MSCs may be formulated with a pharmaceutically acceptable carrier. For example, MSCs may be administered alone or as a component of a pharmaceutical formulation. The subject compounds may be formulated for administration in any convenient way for use in medicine. Pharmaceutical preparations suitable for administration may comprise the MSCs, in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions (*e.g.*, balanced salt solution (BSS)), dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes or suspending or thickening agents.

[049] Concentrations for injections may be at any amount that is effective and, for example, substantially free of hESCs. For example, the pharmaceutical preparations may comprise the numbers and types of MSCs described herein. In a particular embodiment, the pharmaceutical preparations may comprise at least 1×10^6 MSCs derived from hemangioblasts for systemic administration or as few as 1×10^4 MSCs for local administration.

Diseases and Conditions Treated using MSCs derived from hemangioblasts

[050] MSCs have shown be therapeutic for a variety of diseases and conditions. In particular, MSCs migrate to injury sites, exert immunosuppressive effects, and facilitate repair of damaged tissues. Systemic uses of MSCs include, but are not limited to, improving HSC engraftment, suppressing graft-versus-host disease (GvHD), and alleviating debilitating symptoms of autoimmune diseases (e.g., MS, Lupus, Crohn's or other irritable bowel diseases, etc.). Local uses of MSCs include, but are not limited to, reducing inflammation in chronic eye diseases (e.g., retinal degeneration, glaucoma, and uveitis), repairing damaged tissue and reduction of inflammation and pain in osteoarthritis, and repairing damaged tissue in acute

myocardial infarction. As a further example, MSCs may be administered in combination with an allogeneic transplanted cell or tissue (e.g., a preparation comprising cells that have been differentiated from ES cells, such as retinal pigment epithelium (RPE) cells, oligodendrocyte precursors, retinal, corneal, muscle such as skeletal, smooth, or cardiac muscle or any combination thereof, or others) thereby decreasing the likelihood of an immune reaction against the transplanted cell or tissue and potentially avoiding the need for other immune suppression.

[051] MSCs derived from hemangioblasts described herein may be used in similar applications. As such, the disclosure relates to methods of treatment and prevention of various diseases and conditions by administering MSCs derived from hemangioblasts including, but not limited to, multiple sclerosis, systemic sclerosis, hematological malignancies, myocardial infarction, organ transplantation (e.g., kidney) rejection, chronic allograft nephropathy, cirrhosis (e.g., decompensated cirrhosis), liver failure, heart failure, GvHD, tibial fracture (e.g., distal tibial fractures), left ventricular dysfunction, leukemia, myelodysplastic syndrome, Crohn's disease (e.g., moderate to severe), diabetes (e.g., Type I diabetes mellitus), chronic obstructive pulmonary disease, osteogenesis imperfecta, homozygous familial hypocholesterolemia, treatment following meniscectomy, adult periodontitis, vasculogenesis in patients with severe myocardial ischemia, spinal cord injury, osteodysplasia, critical limb ischemia (e.g., in diabetes mellitus), diabetic foot disease, primary Sjogren's syndrome, osteoarthritis, cartilage defects (e.g., articular cartilage defects), multisystem atrophy, amyotropic lateral sclerosis, cardiac surgery, refractory systemic lupus erythematosis, living kidney allografts, nonmalignant red blood cell disorders, thermal burn, Parkinson's disease, microfractures (e.g., in patients with knee articular cartilage injury of defects), epidermolysis bullosa, severe coronary ischemia, idiopathic dilated cardiomyopathy, osteonecrosis femoral head, lupus nephritis, bone void

defects, ischemic cerebral stroke, after stroke, acute radiation syndrome, pulmonary disease, arthritis, bone regeneration, or combinations thereof.

Treatment using MSCs derived from hemangioblasts

[052] The MSCs and pharmaceutically preparations comprising MSCs described herein may be used for cell-based treatments. In particular, the disclosure provides methods for treating or preventing the diseases and conditions described herein comprising administering an effective amount of a pharmaceutical preparation comprising MSCs, wherein the MSCs are derived from hemangioblasts *in vitro*.

[053] The MSCs of the disclosure may be administered using modes known in the art including, but not limited to, intravenous injection (e.g., intramyocardial, transendocardial, intravitreal, intramuscular, etc), local implantation, etc. depending on the particular disease or condition to be treated.

[054] The particular treatment regimen, route of administration, and adjuvant therapy may be tailored based on the particular condition, the severity of the condition, and the patient's overall health. Administration of the pharmaceutical preparations comprising MSCs may be effective to reduce the severity of the symptoms and/or to prevent further degeneration in the patient's condition.

[055] The method of treatment may comprise the administration of a single dose of MSCs. Alternatively, the methods of treatment described herein may comprise a course of therapy where MSCs are administered multiple times over some period to time. Exemplary courses of treatment may comprise weekly, biweekly, monthly, quarterly, biannually, or yearly treatments. Alternatively, treatment may proceed in phases whereby multiple doses are required initially (*e.g.*, daily doses for the first week), and subsequently fewer and less frequent doses are needed.

[056] In one embodiment, MSCs may be delivered one or more times periodically throughout the life of a patient. For example, the MSCs may be delivered once per year, once every 6–12 months, once every 3-6 months, once every 1-3 months, or once every 1-4 weeks. Alternatively, more frequent administration may be desirable for certain conditions or disorders. If administered by an implant or device, the MSCs may be administered one time, or one or more times periodically throughout the lifetime of the patient, as necessary for the particular patient and disorder or condition being treated. Similarly contemplated is a therapeutic regimen that changes over time. For example, more frequent treatment may be needed at the outset (*e.g.*, daily or weekly treatment). Over time, as the patient's condition improves, less frequent treatment or even no further treatment may be needed.

[057] The methods described herein may further comprises the step of monitoring the efficacy of treatment or prevention using methods known in the art.

Kits

[058] The disclosure provides for kits comprising any of the compositions described herein. For example, in one embodiment, the disclosure provides for kits comprising at least 8×10^7, 8.5×10^7, 9×10^7, 9.5×10^7, 1×10^8, 1.25×10^8, or 1.25×10^8 MSCs derived from hemangioblasts. In another embodiment, the disclosure provides for kits comprising pharmaceutical preparations of MSCs derived from hemangioblasts described herein.

Combinations of Various Embodiments and Concepts

[059] It will be understood that the embodiments and concepts described herein may be used in combination. For example, the disclosure provides for a method of generating MSCs comprising generating hemangioblasts from hESCs, culturing the hemangioblasts for at least six days, harvesting the hemangioblasts, re-plating the hemangioblasts on a Matrigel-coated plate, and

culturing the hemangioblasts as described herein for at least fourteen days, wherein the method generates at least 85 million MSCs that are substantially free of hESCs.

Examples

[060] The following examples are not intended to limit the invention in any way.

Example 1 - Generating MSCs from Hemangioblasts

[061] Hemangioblasts were generated from the clinical grade, single-blastomere derived hESC line, MA09 [16], as follows:

[062] First, early-stage embryoid bodies (EBs) were generated from MA09 hESC cultured in serum-free medium supplemented with a combination of morphogens and early hematopoietic cytokines, specifically bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor (VEGF), stem cell factor (SCF), thrombopoietin (Tpo) and fms-related tyrosine kinase 3 ligand (FL). More specifically, hESCs from one well of a 6-well tissue-culture treated plate were plated in one well of a six well ultra low adherence place (Corning) in 3 ml Stemline II medium (Sigma) supplemented with 50 ng/ml of VEGF and 50 ng/ml of BMP-4 (R & D) and incubated at 37° C with 5% CO2. EBs were formed within the first 24 hr. After 40-48 hours, half of the medium (1.5 ml) was replaced with fresh Stemline II medium supplemented with 50 ng/ml of VEGF, 50ng/ml of BMP-4, and 40-45ng/ml bFGF, and incubation continued for an additional 40-48 hours (i.e., 3.5-4 days total).

[063] EBs were dissociated and plated individual cells in serum-free semisolid blast-colony growth medium (BGM). Specifically, EBs were dissociated by 0.05% trypsin-0.53 mM EDTA (Invitrogen) for 2-5 min. The cell suspension was pipeted up and down and then DMEM + 10% FCS was added to inactivate the trypsin. Cells were then passed through a 40μm strainer to obtain a single cell suspension. Cells were then counted and resuspended in Stemline II medium at 1-1.5 X 10^6 cells/ml.

[064] The single cell suspension (0.3 ml, 3to 4.5 X 10^5 cells) was mixed with 2.7 ml of hemangioblast growth medium (H4536 based medium recipe as described above) with a brief vortex, and let stand for 5 min. The cell mixture was then transferred to one well of a six-well ultra low adherence plate by using a syringe (3ml) attached with an 18G needle, and incubated at 37° C with 5% CO2.

[065] Some of the cells developed into grape-like blast colonies (BCs). Specifically, BCs were visible at 3 days (typically contained less than 10 cells at the beginning of day 3), and after 4-6 days, grape-like hES-BCs were easily identified under microscopy (containing greater than 100 cells per BC). The number of BCs present in the culture gradually increased over the course of several days. After 6-7 days, BCs could be picked up using a mouth-glass capillary.

[066] Hemangioblasts can be harvested between day 7-12 of culture and replated onto Matrigel-coated tissue culture plates in α MEM+20% FCS. Flow cytometry analysis shows that expression levels of 5 cell surface markers typically found on MSCs are relatively low in the starting hemangioblast population. (FIG. 2, left panel, average of 4 experiments +/- standard deviation). However, after three weeks of culture in MSC growth conditions, a homogenous adherent cell population arises that stains >90% positive for these 5 characteristic MSC markers (FIG. 2, right panel- 22-23 days, average of 4 experiments +/- standard deviation). Upon MSC culture conditions, the amount of time it takes for differentiating cells to acquire MSC surface markers may vary depending on the specific hESC line used, the day of hemangioblast harvest, and the number of hemangioblasts plated onto Matrigel. In some experiments, markers arise in 90% of the cells by 7-14 days, whereas in other experiments, it may take 22-24 days for this many cells to acquire these MSC markers.

Example 2 - Comparison of Differentiation of hESCs and MSC-derived Hemangioblasts.

[067] This example describes comparison of the differentiation of hESCs into MSCs by two methods: either direct differentiation (in which hESCs were directly plated on gelatin or Matrigel) or the hemangioblast method (in which hESCs were first differentiated into hemangioblasts and then plated on Matrigel, as described in Example 1). Direct differentiation on gelatin gave rise to MSC-like cells, but the cells lacked CD105 expression, suggesting incomplete adoption of MSC fate (FIG. 3, left panel). When hESCs were plated directly on Matrigel, the resulting cells did express CD105 as expected for MSCs (FIG. 3, middle panel). However, compared to MSCs produced by the hemangioblast method, the directly differentiated MSCs cells grew in clumps, were more difficult to disperse when splitting, and did not generate nearly as many MSCs when starting from equivalent numbers of hESCs (FIG. 4).

[068] MSCs differentiated directly from ESCs also took longer to acquire characteristic MSC cell surface markers (FIG. 5). Once MSCs were obtained, extended immunophenotyping shows that MSCs from both methods are positive for other markers typically found on MSCs, such as HLA-ABC, while negative for hematopoiesis-associated markers such as CD34 and CD45 (FIG. 6). These results suggest that use of a hemangioblast-intermediate stage permits robust production of homogeneous MSCs from hESCs. Given these findings, additional studies on MSCs will be conducted with hemangioblast-derived MSCs, not on MSCs generated from the direct hESC-differentiation method.

Example 3 - MSCs Derived from Hemangioblasts Differentiate into Other Cell Types.

[069] MSCs, by definition, should be able to give rise to adipocytes, osteocytes, and chondrocytes. Using standard methods, FIG. 7 shows the ability of hemangioblast-derived

MSCs to differentiate into adipocytes and osteocytes, while FIG. 8 shows their potential to differentiate towards chondrocytes via the expression of chondrocyte-specific genes.

[070] MSCs derived from hemangioblasts are expected to differentiate into adipocytes, osteocytes, and chondrocytes. These differentiation pathways may be examined using methods previously reported in prior art. *See* Karlsson et al, Stem Cell Research 3: 39-50 (2009) (for differentiation of the hemangioblast-derived and direct hESC-derived MSCs into adipocytes and osteocytes (Figure 7)). For chondrocyte differentiation, methods have been adapted from Gong et al, J. Cell. Physiol. 224: 664-671 (2010) to study this process and continue to examine the acquisition of chondrocyte specific genes, (e.g., Aggrecan and Collagen IIa as shown in Figure 8) as well as glycosaminoglycan deposition through safranin O, alcian blue, and/or toluene blue staining. It has been reported in the literature that none of these three cell types, adipocytes, osteocytes, or chondrocytes derived from MSCs will express the immunostimulatory HLA DR molecule (Le Blanc 2003, Gotherstrom 2004, Liu 2006). Immunostaining and/or flow cytometry will be performed on these fully differentiated MSC cell types to confirm these reported observations. This is important to confirm so that differentiation of MSCs in an *in vivo* environment will not induce an immune response from the host recipient. Of these three cell types, chondrogenic differentiation may be of particular interest due to its potential to be used in cartilage replacement therapies for sports injuries, aging joint pain, osteoarthritis, etc. For such therapies, MSCs may not need to be fully differentiated into chondrocytes in order to be used therapeutically.

Example 4 - Confirmation that MSCs Derived from Hemangioblasts are Substantially Free of ESCs

[071] MSCs should also be devoid of the ESC propensity to form teratomas. MSCs were confirmed to contain normal karyotypes (data not shown) by passage 12 (~50 days in culture).

To confirm that the blast-derived MSCs do not contain trace amounts of hESCs, teratoma formation assays will be performed in NOD/SCID mice. 5×10^6 MSCs are injected subcutaneously into the flanks of 4-6 mice. Parent MA09 hESCs will be used as positive controls and the mice will be monitored over the course of 6 weeks to compare teratoma formation in MSC versus hESC-injected mice.

Example 5 - Reduction of EAE Scores by MSCs Derived from Hemangioblasts.

[072] A pilot study to treat experimental autoimmune encephalomyelitis (EAE) on 6-8 weeks of C57BL/6 mice with the hemangioblast-derived hESC-MSCs was conducted. EAE was induced by s.c. injection into the flanks of the mice on day 0 with 100 pL of an emulsion of 50 pg of MOG(35-55) peptide and 250 pg of M. tuberculosis in adjuvant oil (CFA), the mice were also i.p. injected with 500 ng of pertussis toxin. Six days later the mice were i.p. injected with either one million hESC-MSCs in PBS (n = 3) or the vehicle as a control (n = 4). The clinical scores of the animals were recorded for 29 days post the immunization (Fig. 8). A remarkable reduction of the disease scores was observed (Fig. 8).

Example 6 - Confirmation of the Efficacy of hemangioblast-derived hESC-MSCs in EAE Treatment and use of additional animal models of disease

A. Test hESC-MSCs on EAE models in mice confirm their anti-EAE effect.

[073] To confirm the results obtained in Example 5, additional tests are conducted with increased animal numbers, varying cell doses, different administration protocols, and more controls. Clinical score and mortality rate are recorded. The degree of lymphocyte infiltration in the brain and spinal cord of mice will also be assessed. MSC anti-EAE effects is generally thought to involve immunosuppressive activities such as the suppression of Th17 cells and would be expected to reduce the degree of lymphocyte infiltration in the CNS.

B. Compare hESC-MSCs with mouse bone marrow (BM)-MSCs, human BM-MSCs and human UCB-MSCs.

[074] Mouse BM-MSCs were the first to be used for EAE treatment and have been thoroughly studied [1]. hESC-MSCs (given their xenogenic nature) may be directly compared with murine BM-MSCs for anti-EAE efficacy. Human UCB-MSCs have been shown to also possess immunosuppressive activity [19]. The anti-EAE activity of human UCB-MSCs and human BM-MSCs may also be compared with that of hESC-MSCs in the EAE mouse models. The age or passage number of these various cell types may influence their anti-EAE behavior, thus we will also evaluate the consequences of age on the efficacy of MSCs in the EAE mouse model system.

C. Optimize the administration dose, route, and timing of hESC-MSCs.

[075] As shown in FIG. 9, injection of the hESC-MSCs can reduce the scores of EAE as recorded within 29 days after immunization. To study long-term prevention and cure of disease, hESC-MSCs may be administered at various doses, routes, and times.

[076] MSCs have been generated from H1gfp hESCs and confirmed that they still express GFP in the MSC state. EAE mice can be injected with these GFP+ hESC-MSCsand their distribution can be tracked *in vivo* by using a Xenogen In Vivo Imaging System. Through these approaches, various administration doses, routes, and timing of hESC-MSCs will be analyzed and provide information as to the mechanism of action for MSCs anti-EAE activity (ie, paracrine or endocrine effects), longevity of the MSCs within the mice and MSC biodistribution and routes of elimination/clearance.

[077] Based on the results in FIG. 9, the inventor expects to confirm and further characterize the anti-EAE effects of hESC-MSCs in the mouse model. Anti-EAE effects may be

reflected by one or more of reduced clinical scores, increased survival, and/or attenuated lymphocyte infiltration and demyelination of the CNS. Different hESC lines may have different intrinsic abilities to generate MSCs. Therefore, multiple hESC lines may be used in this study and acquisition of MSC markers can be monitored over time and compared for each hESC line. To further reduce variations between experiments with hESC-MSCs, large stocks of frozen hESC-MSCs can be made in aliquots and each stock of aliquots can be used in multiple experiments.

D. Confirm efficacy of hemangioblast-derived MSCs in other disease models.

[078] As mentioned above, MSCs may also have therapeutic activity against other types of autoimmune disorders such as Crohn's disease, ulcerative colitis, and the eye-disorder, uveitis. Animal models for these diseases exist and are well known in the art (*see, e.g.*, Pizarro et al 2003, Duijvestein et al 2011, Liang et al 2011, Copland. et al 2008). *In vivo* studies may be expanded to include an assessment of MSC therapeutic utility in one or more of these animal model systems. Such models may allow us to examine the cytokine secretion profile of human MSCs by isolating and screening the serum of injected animals for human cytokines. Particularly, the uveitis model may be useful as a local intraviteal injection may allow us to study the effects of MSCs in a non-systemic environment.

[079] MSCs may also have great therapeutic utility in treating osteoarthritis conditions, including those that involve loss of articular cartilage and inflammation of the affected joints (Noth et al, 2008). Models for examining osteoarthritis, cartilage loss and joint inflammation are well known in the art (*see, e.g.*, Mobasheri et al 2009). In some of these studies, human BM-MSCs are encapsulated in semi-solid scaffolds or microspheres and transplanted into an affected joint in human subjects to determine if the MSCs have a local, non-systemic therapeutic effect in

terms of reduced inflammation and/or restoration of cartilage (Wakitani et al 2002). Such methods will assist in determining the therapeutic utility of our hESC heamngioblast-derived MSCs for treating degenerative joint conditions.

[080] The life span of injected MSCs is very short [8], which indicates that long-term survival of the transplanted cells is not required. Thus, mitotically-inactivated hESC-MSCs (e.g., irradiated or treated with mitomycin C) may also be tested for an anti-EAE effect or other anti-disease effect in the animal models mentioned above. If so, live hESC-MSCs may not be needed, thus further decreasing the biosafety concern from potential residual hESC contamination in the transplanted hESC-MSCs.

E. Results

[081] MSCs from different donor derive sources (mouse BM-MSCs, human BM-MSCs and human UCB-MSCs) are expected to harbor anti-EAE effects. However, their effects may vary between experiments as the MSCs are from donor-limited sources. In contrast, the hESC-MSCs of the present disclosure may have more consistent effects. Because many cell surface markers are used to characterize MSCs and not every MSC expresses all the markers, a subset of markers, e.g., CD73+ and CD45- may be used in order to compare efficacy of MSCs from different sources.

[082] hESC-MSCs are expected to have therapeutic utility in animal models of Crohn's Disease, ulcerative colitis, and uveitis as these contain autoimmune components and inflammatory reactions.

[083] The irradiated hESC-MSCs may retain, at least partially, the immunosuppressive function since they still secret cytokines and express cell surface markers that are related to the function [29]. Their effect may, however, be decreased due to their shortened life span *in vivo*. If so, the

dose of irradiated cells and administration frequency may be increased to enhance the immunosuppressive function. The irradiated hESC-MSCs may retain, at least partially, the immunosuppressive function since they still secret cytokines and express cell surface markers that are related to the function [29]. Their effect may, however, be decreased due to their shortened life span *in vivo*. If so, the dose of irradiated cells and administration frequency may be increased to enhance the immunosuppressive function.

Example 7 - Investigation of functional components of hESC-MSCs

[084] MSCs may be defined as plastic adherent cells that express the following cell surface markers: CD105, CD73, CD29, CD90, CD166, CD44, CD13, and HLA-class I (ABC) while at the same time being negative for CD34, CD45, CD13, and CD31 when cultured in an uninduced state (eg, culture in regular αMEM+20%FCS with no cytokines). Under these conditions, they must express intracellular HLA-G and be negative for CD40 and HLA class II (DR). Functionally, such cells must also be able to differentiate into adipocytes, osteocytes, and chondrocytes as assessed by standard *in vitro* culture assays. After 7 days stimulation with interferon gamma (IFNγ), MSCs should express HLA-G on their cell surface as well as CD40 and HLA-class II (DR) on their cell surface. Despite these requirements, MSCs derived from any source may contain some heterogeneity and due to the pluripotency of hESCs it is possible that MSC cultures derived from hESCs may contain cells of any lineage from the three germ layers. While the culture system described herein indicated that >90% of cells routinely display the above mentioned immunophenotype and functional characteristics, small subpopulation(s) of cells within the MSC culture may exist that lack expression of one or more of the MSC cell surface markers or express one or more of the markers that should be absent. The extent of such subpopulations within our MSC cultures will be examined to determine the degree of

contaminating heterogeneity. Multicolor flow cytometry (8+ colors simultaneously) can be performed on a BD LSR II flow cytometer in order to determine the overlap between the above mentioned markers. This may also help pinpoint the exact cell surface marker profile that is required for the greatest immunosuppressive activity.

A. Characterize the differentiation stage, subpopulations, and activation status of hESC-MSCs in relevance to their immunosuppressive effects.

[085] There is a large time window (e.g., at least from day 14 to 28 in the MSC differentiation medium) to harvest hESC-MSCs (*see, e.g.*, FIG. 1). Several studies have indicated that MSCs tend to lose their immunosuppressive functions and may senesce as they are continually passaged and age during long culture periods. As such, the cells may be harvested at different time points activity in order to determine is a specific number of days in MSC medium affords greater immunosuppressive activity. Indeed, MSCs collected at an early timepoint (e.g., 14 days in MSC culture conditions) may contain precursor cells that have not yet fully acquired all of the characteristic MSC cell surface markers but that harbor highly potent immunosuppressive effects. To define potentially useful MSC precursor populations, the expression of a wide range of cell surface markers are being tracked throughout the MSC differentiation process, from day 7 through day 28. It has been observed that at least 50% of the culture will acquire the cell surface marker CD309 (other names include VEGFR2, KDR) within 14 days of MSC culture conditions. CD309 is largely absent from the starting hemangioblast population (Figure 10, first time point, MA09 hemangioblasts harvested at d7 and 8), but rises within the first two weeks of MSC culture conditions and then declines again back to less than 5% of the cells by day 28 (Figure 10, second, third, and fourth time points). This pattern has been found to occur not only with MA09 hemangioblast-derived MSCs but also with those from

MA01, H1gfp, and H7 hESCs. In these experiments, hemangioblasts are routinely negative (less than 5% of cells stain positive) for CD309 regardless of their harvest date (day 6-14). However, the percentage of developing MSCs that acquire CD309 expression may be reduced when developing from older hemangioblasts (e.g., d10 or d12 blasts). In a similar fashion, it has been observed that the expansion properties of hemangioblast-derived MSCs may differ depending on the harvest date of hemangioblasts. MSCs developing from younger hemangioblasts (day 6 or 7) do not continue to expand as robustly as MSCs developing from older (d8-12) hemangioblasts. The optimal date of hemangioblast harvest may be an intermediate one (day 8-10) as they may allow adequate acquisition of CD309 as a surrogate marker of MSC development while still maintaining a robust ability to expand through day 28 and beyond. Work is ongoing to optimize these aspects of MSC precursor development.

[086] Except CD105 and CD73 that have proved the most typical markers for MSCs, many other cell surface molecules not mentioned above such as CD49a, CD54, CD80, CD86, CD271, VCAM, and ICAM have also been proposed or used as MSC markers [22]. It is therefore possible that hESC-MSCs may contain subpopulations that express various combinations of other markers during the differentiation from HB, which may possess varying immunosuppressive activities. Subpopulations may be sorted (e.g., using FACS) based one or more markers (individually or in combination) for analysis to compare their immunosuppressive activity using *in vitro* or *in vivo* methods.

B. Optimize differentiation and expansion conditions to obtain large quantities of functional hESC-MSCs.

[087] While preliminary experiments have indicated that MSCs may be maintained in IMDM + 10% heat-inactivated human serum, we have not yet tested their derivation in this

medium. Different culture conditions will be tested to determine whether substituting culture components (eg, base medium, serum source, serum replacement products, human serum platelet lysate) may enrich the effective subpopulations described herein. Different basal medium including animal-free and a defined culture (without FBS) system to culture hESCs and prepare MSCs will be evaluated. Specifically, StemPro® MSC SFM from Invitrogen and the MSCM bullet kit from Lonza will be used to examine if a serum-free defined culture system would generate hESC-MSCs with desired quality and quantity. Also, various growth factors such as FGFs, PDGF, and TGFI3, as well as small chemicals that regulate signaling pathways or cell structures, may be used to enhance the quality and quantity of hESC-MSCs.

C. Results

[088] The hESC-MSCs expressing the typical markers CD73 (ecto-5'-nucleotidase [26]), CD54 (ICAM-1, an integrin ligand [27]), and CD271 (an inhibitor of MSC differentiation [28]) may possess the strongest immunosuppressive effect *in vitro*.

Example 8 - Investigation of the mechanism of immunosuppression by hESC-MSCs

A. Study how hESC-MSCs may suppress adaptive immune responses mediated by T cells.

[089] A general response of naïve T cells isolated from PBMC is to proliferate when they are induced with mitotic stimulators such as phytohemaglutinin (PHA) or phorbol myristate acetate (PMA)/ionomycin or when they encounter antigen presenting cells (APCs) such as dendritic cells. This is best exemplified by the general proliferation of CD4+ and CD8+ T cells in a mixed leukocyte reaction (MLR) assay. Prior studies indicate that MSCs can suppress T cell proliferation in an MLR assay.

[090] The ability of our hESC-hemangioblast derived MSCs to inhibit T cell proliferation caused by either chemical stimulation (PMA/ionomycin, Figure 11a) or exposure to APCs (dendritic cells, Figure 11b) was examined. It was observed that MSCs dampened the proliferative response of T cells due to either chemical stimulation or co-culture with APCs and that this suppression occurred in a dose dependent manner (Figure 11b, graph on right) Moreover, it was found that mitotically inactivated MSCs (Figure 11b) were able to suppress T cell proliferation to an equivalent degree as live MSCs, suggesting that mitotically inactivated MSCs may indeed be useful *in vivo* for immunosuppression.

[091] Various functional subsets of T cells exist and they carry out specific roles involved in proinflammatory responses, anti-inflammatory responses, or induction of T cell anergy. Regulatory T cells (Tregs) can be thought of as naturally occurring immunosuppressive T cells and in a normal setting, are responsible for dampening hypersensitive auto-reactive T cell responses. They usually represent only a small proportion of the body's T cells but their prevalence can be influenced by various environmental factors. MSCs have been shown to induce peripheral tolerance through the induction of Treg cells [33-35].

[092] In a short, 5 day co-culture assay, it was found that, similar to prior studies, the hemangioblast-derived MSCs were able to increase the percentage of CD4/CD25 double positive Tregs that are induced in response to IL2 stimulus (Figure 12a). Co-culture of a mixed T cell population from non-adherent peripheral blood mononuclear cells (PBMCs) with MSCs (at a ratio of 10 PBMCs:1 MSC) shows that Treg induction nearly doubled when MSCs were included in the IL2 induced culture. This degree of Treg induction is similar to that observed in the highly cited Aggarwal et al study published in Blood, 2005. The amount of FoxP3 induced within the CD4/CD25 double positive population will be examined to confirm that these are indeed true

Tregs. Intracellular flow cytometry, RT-PCR, and Western blot analyses will all be employed to study FoxP3 induction in the absence and presence of MSCs in the IL2-induced T cell cultures. Both non-adherent PBMCs and purified CD4+ T cell populations may be used to study Treg induction in these assays.

[093] Th1 and Th17 cells are thought to play important roles in MS and in other autoimmune diseases. The differentiation and function of Th1 and Th17 CD4+ T cells will be analyzed first and foremost using *in vitro* assays; they may also be examined in the EAE model or in other animal models we may employ. The effects of MSCs on Th1 induction *in vitro* have begun to be examined. Culture conditions that promote Th1 specification from naïve CD4+ T cells are known in the field (Aggarwal et al). These culture conditions (which include anti-CD3, anti-CD28, and anti-CD4 antibodies together with human IL3 and IL12) have been employed to induce Th1 cells from naïve, non-adherent PBMCs in the absence or presence of MSCs (10 PBMCs:1 MSC). After 48 hours of co-culture, non-adherent cells were isolated, rinsed, and stimulated with PMA/ionomycin for 16 hours in a new well. After the 16 hour induction, supernants were collected and analyzed for secretion of the Th1 cytokine, IFNγ. As anticipated, it was found that the PBMCs cultured with MSCs in the 48 hr Th1 inducing conditions did not produce as much IFNγ as those cultured without MSCs. This indicates that MSCs can suppress a major Th1 cell function, i.e., IFNγ secretion. (Figure 12b) Similar studies will be performed by differentiating Th17 cells *in vitro* and determining the effects of MSCs on pro-inflammatory IL17 secretion using an ELISA assay on culture supernatants.

[094] Th2 cells are known to secrete cytokines that have anti-inflammatory effects, such as IL4. MSCs may be able to enhance Th2 differentiation and secretion of IL4. Similar to the experiment described above for Th1 cells, Th2 inducing conditions will be used in a 48 hour

culture system to stimulate Th2 differentiation from naïve PBMC containing T cells. The effects of MSC co-culture on IL4 secretion will be examined using an ELISA assay.

[095] Recently, studies have suggested that CD8 T cells also play a pivotal role in EAE models and the underlying mechanism of MS[30]. The inventor will examine if co-culture with hESC-MSCs *in vitro* may affect the function of CD8 T cells. To do this, non-adherent PBMCs or purified CD8+ T cells will be exposed to EAE-associated MBP110-118 peptide through the use of APCs. This will cause an antigen-specific CD8+ T cell population to emerge and such a population can be expanded using CD3/CD28 expander beads (Invitrogen). Existence of the angiten-specific CD8+ T cells can be verified using a pentamer reagent specific for the MBP-peptide (Proimmune) in flow cytometry. Re-stimulation with MBP110-118-loaded APCs will be performed in order to induce an antigen specific immune response, which includes both expansion of the antigen-specific CD8+ T cells and secretion of IFNγ. The response from T cells cultured in the absence or presence of MSCs will be compared to determine if the MSCs can suppress the induction of these cytotoxic EAE-associated antigen specific T cells. Pentamer specific flow cytometry, BrdU incorporation, and ELISA assays will be employed for this purpose.

B. Determine if inflammatory factors and inter-cellular adhesion molecules (ICAMs) contribute to the immunosuppresive effect of hESC-MSCs.

[096] It has been shown that TGFbeta, PGE2, IDO, nitric oxide (NO), and ICAMs are important for the immunosuppressive function of MSCs [7]. The secretion of these molecules and expression of ICAMs by hESC-MSCs will be examined using ELISA assays and flow cytometry.

[097] It has been shown that the pro-inflammatory cytokine, IFNγ is required for the activation of MSCs [23], and various agonists for Toll-like receptors (TLRs) such as LPS and poly(I:C) can

induce different subsets of MSCs [24]. For example, it has recently been shown that IFNγ-activated MSCs have greater therapeutic efficacy in a mouse model of colitis than do untreated MSCs (Duijvestein et al 2011). The effects of IFNγ on MSC properties have begun to be examined. hESC-MSCs have been treated *in vitro* with IFNγ for up to seven days and striking changes in cell surface marker expression have resulted. These findings are consistent with observations made in previous studies (Gotherstrom et al 2004, Rasmusson et al 2006, Newman et al 2009) and confirm that the hemangionblast derived hESC-MSCs function similarly to MSCs isolated from the body. For example, in a resting state, MSCs typically do not express much (<10%) HLA G on their cell surface while they do harbor intracellular stores of this special class of immunotolerant HLA marker. Upon 7 days IFNγ treatment, HLA G can be readily detected at the cell surface (Figure 13) and may also be induced to be secreted (not yet tested). Additionally, IFNγ treatment causes an upregulation of CD40 expression and HLA DR expression at the cell surface (Figure 13). These changes are proposed to enhance their immunosuppressive effects. For example, we will determine if pretreatment of MSCs with IFNγ enhances their ability to induce Treg populations, to suppress Th1 secretion of IFNγ, or to enhance IL4 secretion from Th2 cells by using in vitro co-culture assays described above. IFNγ may also influence the ability of MSCs to inhibit general T cell proliferation in MLR assays. The effects of TNFα, LPS, and/or poly I:C on these types of MSC immunosuppressive properties may also be tested.

C. Results

[098] It is expected that the CD4/CD25 double positive population of Tregs induced by MSCs will also express the transcription factor, FoxP3 as it has been reported that functional Tregs upregulate its expression in response to inducing stimuli.

[099] It is expected that MSCs will inhibit, to some degree the pro-inflammatory secretion of IL17 by Th17 cells and that MSCs can also significantly enhance IL4 secretion by anti-inflammatory Th2 cells. Such observations have been made in previous studies and will assist in confirming the true functionality of the hemangioblast-derived MSCs.

[0100] The hESC-MSCs should inhibit at least partially the antigen-induced activation of CD8+ T cells. The function of NK cells, macrophages, and dendritic cells after hESC-MSC co-culture may also be examined. The effects of hESC-MSCs on maturation, cytotoxicity, and/or specific cytokine production by these other types of immune cells will be examined.

References Cited

1. Zappia, E., et al., *Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy.* Blood, 2005. 106(5): p. 1755-61.
2. Gerdoni, E., et al., *Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis.* Ann Neurol, 2007. 61(3): p. 219-27.
3. Lanza, C., et al., *Neuroprotective mesenchymal stem cells are endowed with a potent antioxidant effect in viva* J Neurochem, 2009. 110(5): p. 1674-84.
4. Rafei, M., et al., *Allogeneic mesenchymal stem cells for treatment of experimental autoimmune encephalomyelitis.* Mol Ther, 2009. 17(10): p. 1799-803.
5. Rafei, M., et al., *Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th 17 T cells in a* CC *chemokine ligand 2-dependent manner.* J Immunol, 2009. 182(10): p. 5994-6002.
6. Constantin, G., et al., *Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis.* Stem Cells, 2009. 27(10): p. 2624-35.
7. Uccelli, A. and D.J. Prockop, *Why should mesenchymal stem cells (MSCs) cure autoimmune diseases?* Curr Opin Immunol, 2010. 22(6): p. 768-74.
8. Ohtaki, H., et al., *Stem/progenitor cells from bone marrow decrease neuronal death in global ischemia by modulation of inflammatory/immune responses.* Proc Natl Acad Sci U S A, 2008. 105(38): p. 14638-43.
9. Barberi, T., et al., *Derivation of multipotent mesenchymal precursors from human embryonic stem cells.* PLoS Med, 2005. 2(6): p. e161.
10. Hwang, N.S., et al., *In vivo commitment and functional tissue regeneration using human embryonic stem cell-derived mesenchymal cells.* Proc Natl Acad Sci U S A, 2008. 105(52): p. 20641-6.
11. Olivier, E.N., A.C. Rybicki, and E.E. Bouhassira, *Differentiation of human embryonic stem cells into bipotent mesenchymal stem cells.* Stem Cells, 2006. 24(8): p. 1914-22.
12. Brown, S.E., W. Tong, and P.H. Krebsbach, *The derivation of mesenchymal stem cells from human embryonic stem cells.* Cells Tissues Organs, 2009. 189(1-4): p. 256-60.
13. Karlsson, C., Emanuelsson, K., Wessberg, F., Kajic, K., Axell, M. Z., Eriksson, P. S., Lindahl, A., Hyllner, J., and Strehl, R., *Human embryonic stem cell-derived mesenchymal progenitors-Potential in regenerative medicine.* Stem Cell Res., 2009. 3(1): 39-50.
14. Lu, S. J., Feng, Q., Caballero, S., Chen, Y., Moore, M. A., Grant, M. B., and Lanza, R., *Generation of functional hemangioblasts from human embryonic stem cells*, Nat. Methods 4 (2007) 501-509.
15. Lu, S. J., Luo, C., Holton, K., Feng, Q., Ivanova, Y., and Lanza, R., *Robust generation of hemangioblastic progenitors from human embryonic stem cells*, Regen. Med. 3 (2008)

693-704.

16. Klimanskaya, I., Chung, Y., Becker, S., Lu, S.-J., and Lanza, R., *Human embryonic stem-cell lines derived from single blastomeres*, Nature 444 (2006) 481-485.

17. Madsen, L.S., et al., *A humanized model for multiple sclerosis using HLA-DR2 and a human T-cell receptor.* Nat Genet, 1999. 23(3): p. 343-7.

18. Stromnes, I.M. and J.M. Goverman, *Passive induction of experimental allergic encephalomyelitis.* Nat Protoc, 2006. 1(4): p. 1952-60.

19. Liang, J., et al., *Allogeneic mesenchymal stem cells transplantation in treatment of multiple sclerosis.* Mult Scler, 2009. 15(5): p. 644-6.

20. Costa, M., et al., *The hESC line Envy expresses high levels of GFP in all differentiated progeny.* Nat Methods, 2005. 2(4): p. 259-60.

21. Pomper, M.G., et al., *Serial imaging of human embryonic stem-cell engraftment and teratoma formation in live mouse models.* Cell Res, 2009. 19(3): p. 370-9.

22. Phinney, D.G. and D.J. Prockop, *Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views.* Stem Cells, 2007. 25(11): p. 2896-902.

23. Ryan, J.M., et al., *Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells.* Clin Exp Immunol, 2007. 149(2): p. 353-63.

24. DelaRosa, 0. and E. Lombardo, *Modulation of adult mesenchymal stem cells activity by toll-like receptors: implications on therapeutic potential.* Mediators Inflamm, 2010. 2010: p. 865601.

25. English, K., et al., *IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells.* Immunol Lett, 2007. 110(2): p. 91-100.

26. Barry, F., et al., *The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells.* Biochem Biophys Res Commun, 2001. 289(2): p. 519-24.

27. Alhadlaq, A. and J.J. Mao, *Mesenchymal stem cells: isolation and therapeutics.* Stem Cells Dev, 2004. 13(4): p. 436-48.

28. Mikami, Y., et al., *CD271/p75NTR inhibites the differentiation of mesenchymal stem cells into osteogenic, adipogenic, chondrogenic, and myogenic lineages.* Stern Cells Dev, 2010.

29. Di Nicola, M., et al., *Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli.* Blood, 2002. 99(10): p. 3838-43.

30. Johnson, T.A., F.R. Jirik, and S. Fournier, *Exploring the roles of CD8(+) T lymphocytes in the pathogenesis of autoimmune demyelination.* Semin Immunopathol, 2010. 32(2): p. 197-209.

31. Huseby, E.S., C. Ohlen, and J. Goverman, *Cutting edge: myelin basic protein-specific cytotoxic T cell tolerance is maintained in vivo by a single dominant epitope in H-2k mice.* J Immunol, 1999. 163(3): p. 1115-8.

32. Tang, Q. and J.A. Bluestone, *Regulatory T-cell physiology and application to treat autoimmunity.* Immunol Rev, 2006. 212: p. 217-37.

33. Boumaza, I., et al., *Autologous bone marrow-derived rat mesenchymal stem cells promote PDX-1 and insulin expression in the islets, alter T cell cytokine pattern and preserve regulatory T cells in the periphery and induce sustained normoglycemia.* J Autoimmun, 2009. 32(1): p. 33-4

34. Maccario, R., et al., *Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype.* Haematologica, 2005. 90(4): p. 516-25.

35. Locatelli, F., R. Maccario, and F. Frassoni, *Mesenchymal stromal cells, from indifferent spectators to principal actors. Are we going to witness a revolution in the scenario of allograft and immune-mediated disorders?* Haematologica, 2007. 92(7): p. 872-7.

36. Wan, Y.Y. and R.A. Flavell, *Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter.* Proc Natl Acad Sci U S A, 2005. 102(14): p. 5126-31.

37. Duijvestein et al. Stem Cells 29 (10): 1549-1558 (2011)

38. Liang et al. Cell Transplant 20 (9): 1395-1408 (2011)

39. Pizarro et al. Trends Mol Med 9 (5): 218-222 (2003)

40. Copland et al. IOVS 49 (12): 5458-5465 (2008)

41. Wakitani et al. Osteoarthritis and Cartilage 10: 199-206 (2002)

42. Mobasheri et al. Histol. Histopathol 24 (3): 347-366 (2009)

43. Noth et al. Nat Clin Pract Rheumato 4(7): 371-380 (2008)

44. Gotherstrom et al. Am J Obstet Gynecol 190(1): 239-45 (2004)

45. Rasmusson et al. Exp. Cell Research 312(12): 2169-79 (2006)

46. Newman et al. Inflamm Allergy Drug Targets 8(2): 110-123 (2009)

47. Le Blanc et al. Exp. Hematol. 31: 890-896 (2003)

48. Liu et al. J. of Immunol. 176: 2864-2871 (2006)

Each document cited herein (e.g., U.S. patents, U.S. published applications, non-patent literature, etc.) is hereby incorporated by reference in its entirety.

Claims

1. A method for generating mesenchymal stromal cells comprising culturing hemangioblasts.

2. The method of claim 1, wherein said hemangioblasts are cultured in feeder-free conditions.

3. The method of claim 1 or 2, wherein said hemangioblasts are plated on a matrix.

4. The method of claim 3, wherein said matrix comprises one or more of: transforming growth factor beta (TGF-beta), epidermal growth factor (EGF), insulin-like growth factor 1, bovine fibroblast growth factor (bFGF), and/or platelet-derived growth factor (PDGF).

5. The method of claim 3 or 4, wherein said matrix is selected from the group consisting of: laminin, fibronectin, vitronectin, proteoglycan, entactin, collagen, collagen I, collagen IV, heparan sulfate, and Matrigel (a soluble preparation from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells), a human basement membrane extract, and any combination thereof.

6. The method of any one of claims 3-5, wherein said matrix is Matrigel.

7. The method of any one of claims 3-6, wherein said matrix is of human or non-human animal.

8. The method of claim 7, wherein said matrix is of bovine, mouse or rat origin.

9. The method of any foregoing claim, wherein said mesenchymal stromal cells are human.

10. The method of any foregoing claim, wherein said hemangioblasts are cultured in a medium comprising αMEM.

11. The method of any foregoing claim, wherein said hemangioblasts are cultured in a medium comprising serum or a serum replacement.

12. The method of any foregoing claim, wherein said hemangioblasts are cultured in a medium comprising, consisting of, or consisting essentially of αMEM supplemented with 20% fetal calf serum.

13. The method of any one of claims 3-12, wherein said hemangioblasts are cultured on said matrix for at least 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 days.

14. The method of any foregoing claim, wherein said hemangioblasts are differentiated from ESCs.

15. The method of claim 14, wherein said ESCs are iPS cells.

16. The method of claim 15, wherein said ESCs comprise MA09, H7, H9, MA01, HuES3, or H1gfp cells.

17. The method of claim 15, wherein said ESCs are derived from one or more inner cell mass cells or one or more blastomeres.

18. The method of any one of claims 15-17, wherein said hemangioblasts are differentiated from ESCs by a method comprising (a) culturing said ESCs to form embryoid bodies.

19. The method of claim 18, wherein said embryoid bodies are formed in the presence of vascular endothelial growth factor (VEGF) and/or bone morphogenic protein 4 (BMP-4).

20. The method of claim 19, wherein said VEGF and BMP-4 are added to the cell culture of step (a) within 0-48 hours of initiation of said cell culture, wherein said VEGF is optionally added at a concentration of 20-100 nm/mL and wherein said BMP-4 is optionally added at a concentration of 15-100 ng/mL.

21. The method of any one of claims 18-20, wherein said hemangioblasts are differentiated from ESCs by a method further comprising: (b) culturing said embryoid bodies in the presence

of at least one growth factor in an amount sufficient to induce the differentiation of said embryoid bodies into hemangioblasts.

22. The method of claim 21, wherein said at least one growth factor added in step (b) comprises one or more of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and bone morphogenic protein 4 (BMP-4), stem cell factor (SCF), Flt 3L (FL), thrombopoietin (TPO), and/or tPTD-HOXB4.

23. The method of claim 21 or 22, wherein one or more of said at least one growth factor added in step (b) is added to said culture comprising embryoid bodies within 48-72 hours from the start of step (a).

24. The method of claim 23, wherein said at least one factor added in step (b) comprises one or more of bFGF, VEGF, BMP-4, SCF, FL, and/or tPTD-HOXB4.

25. The method of any one of claims 22-24, wherein the concentration in step (b) of VEGF is 20-100 ng/ml, BMP-4 is 15-100 ng/ml, SCF is 20 or 50 ng/ml, FL is 10-50 ng/ml, TPO is 20 or 50 ng/ml, and/or tPTD-HOXB4 is 1.5-5 U/ml.

26. The method of any one of claims 21-25, wherein said at least one growth factor is added to said culture multiple times throughout step (b).

27. The method of any one of claims 18-26, wherein said embryoid bodies are dissociated, optionally into single cells.

28. The method of claim 27, further comprising: (c) culturing said hemangioblasts in a medium comprising at least one additional growth factor, wherein said at least one additional growth factor is in an amount sufficient to expand human hemangio-colony forming cells in said culture.

29. The method of claim 28, wherein said at least one additional growth factor comprises one or more of: insulin, transferrin, granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), stem cell factor (SCF), vascular endothelial growth factor (VEGF), bone morphogenic protein 4 (BMP-4), and/or tPTD-HOXB4.

30. The method of claim 29, wherein said at least one additional growth factor comprises insulin, transferrin, GM-CSF, IL-3, IL-6, G-CSF, EPO, SCF, VEGF, BMP-4, or tPTD-HOXB4.

31. The method of any one of claims 29-30 wherein the concentration in step (d) of insulin is about 10 μg/ml, transferrin is about 200 μg/ml, GM-CSF is about 20 μg/ml, IL-3 is about 20 ng/ml, IL-6 is about 10-20 ng/ml, G-CSF is about 20 ng/ml, EPO is about 3-6 U/ml, SCF is about 20-50 ng/ml, VEGF is about 20-100 ng/ml, BMP-4 is about 15-100 ng/ml, and/or tPTD-HOXB4 is about 1.5-5U/ml.

32. The method of any one of claims 28-31, wherein said at least additional one growth factor is added to said culture multiple times throughout step (d).

33. The method of any one of claims 18-32, wherein said medium in step (a), (b) and/or (d) is a serum-free medium.

34. The method of any foregoing claim, wherein at least 80, 85, 90, 95, 100, 125, or 150 million mesenchymal stromal cells are generated.

35. The method of any one of claims 11 to 34, wherein said hemangioblasts are harvested after at least 6, 7, 8, 9, 10, 11, 12, 13, or 14 days of starting to induce differentiation of said ESCs.

36. The method of claim 35, wherein said mesenchymal stromal cells are generated within at least 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 days of starting to induce differentiation of said ESCs.

37. The method of any foregoing claim, wherein at least 80, 85, 90, 95, 100, 125, or 150 million mesenchymal stromal cells are generated from about 200,000 hemangioblasts within at least 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 days of culturing the hemangioblasts.

38. The method of any foregoing claim, wherein said mesenchymal stromal cells comprise less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%, 0.009%, 0.008%, 0.007%, 0.006%, 0.005%, 0.004%, 0.003%, 0.002%, 0.001%, 0.0009%, 0.0008%, 0.0007%, 0.0006%, 0.0005%, 0.0004%, 0.0003%, 0.0002%, or 0.0001% human embryonic stem cells.

39. The method of any foregoing claim, wherein said mesenchymal stromal cells are substantially purified with respect to human embryonic stem cells and comprise at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% mesenchymal stromal cells.

40. The method of any foregoing, wherein said mesenchymal stromal cells do not form teratomas when introduced into a host animal, which is optionally an immunocompromised host animal.

41. Mesenchymal stromal cells derived from hemangioblasts by the method of any foregoing claim.

42. Mesenchymal stromal cells derived from hemangioblasts.

43. The mesenchymal stromal cells of claim 41 or 42, wherein at least 50% of said mesenchymal stromal cells are positive for CD105 or CD73 after 15 days of culture.

44. The mesenchymal stromal cells of any one of claims 41-43, wherein at least 80% said mesenchymal stromal cells are positive for CD105 and CD73 within 20 and 21 days of culture, respectively.

45. The mesenchymal stromal cells of any one of claims 41-44, comprising at least 80, 85, 90, 95, 100, 125, or 150 million mesenchymal stromal cells, wherein said mesenchymal stromal cells are generated from about 200,000 hemangioblasts within at least 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 days of culturing the hemangioblasts.

46. The mesenchymal stromal cells of any one of claims 41-45, wherein said mesenchymal stromal cells comprise less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%, 0.009%, 0.008%, 0.007%, 0.006%, 0.005%, 0.004%, 0.003%, 0.002%, 0.001%, 0.0009%, 0.0008%, 0.0007%, 0.0006%, 0.0005%, 0.0004%, 0.0003%, 0.0002%, or 0.0001% human embryonic stem cells.

47. The mesenchymal stromal cells of any one of claims 41-45, wherein said mesenchymal stromal cells are substantially purified with respect to human embryonic stem cells and comprise at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% mesenchymal stromal cells.

48. The mesenchymal stromal cells of any one of claims 41-47, wherein said mesenchymal stromal cells do not form teratomas when introduced into a host animal, which is optionally an immunocompromised host animal.

49. The mesenchymal stromal cells of any one of claims 41-48, wherein said mesenchymal stromal cells are capable of undergoing at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, or more population doublings in culture.

50. The mesenchymal stromal cells of any one of claims 41-49, wherein said mesenchymal stromal cells (a) do not clump or clump substantially less than mesenchymal stromal cells derived directly from hESCs; (b) more easily disperse when splitting compared to mesenchymal stromal cells derived directly from hESCs; (c) are greater in number than mesenchymal stromal cells derived directly from hESCs when starting with equivalent numbers of hESCs; and/or (d) acquire characteristic mesenchymal cell surface markers faster than mesenchymal stromal cells derived directly from hESCs.

51. A kit comprising the mesenchymal stromal cells of any of claims 41-50.

52. A pharmaceutical preparation comprising the mesenchymal stromal cells of any of claims 41-50.

53. A method for treating a disease or disorder, comprising administering an effective amount of mesenchymal stromal cells derived from hemangioblasts to a subject in need thereof.

54. The method of claim 53, wherein the disease or disorder is multiple sclerosis, systemic sclerosis, hematological malignancies, myocardial infarction, organ transplantation rejection, chronic allograft nephropathy, cirrhosis, liver failure, heart failure, GvHD, tibial fracture, left ventricular dysfunction, leukemia, myelodysplastic syndrome, Crohn's disease, diabetes, chronic obstructive pulmonary disease, osteogenesis imperfecta, homozygous familial hypocholesterolemia, treatment following meniscectomy, adult periodontitis, vasculogenesis in patients with severe myocardial ischemia, spinal cord injury, osteodysplasia, critical limb ischemia, diabetic foot disease, primary Sjogren's syndrome, osteoarthritis, cartilage defects, multisystem atrophy, amyotropic lateral sclerosis, cardiac surgery, refractory systemic lupus erythematosis, living kidney allografts, nonmalignant red blood cell disorders, thermal burn, Parkinson's disease, microfractures, epidermolysis bullosa, severe coronary ischemia, idiopathic

dilated cardiomyopathy, osteonecrosis femoral head, lupus nephritis, bone void defects, ischemic cerebral stroke, after stroke, acute radiation syndrome, pulmonary disease, arthritis, bone regeneration, or combinations thereof.

55. The method of claim 53, wherein the disease or disorder is uveitis.

56. The method of claim 53, wherein said disease or disorder is an autoimmune disorder or an immune reaction against allogeneic cells.

57. The method of claim 56, wherein the autoimmune disorder is multiple sclerosis.

58. A method of treating bone loss or cartilage damage comprising administering an effective amount of mesenchymal stromal cells derived from hemangioblasts to a subject in need thereof.

59. The method of any one of claims 53-58, wherein the mesenchymal stromal cells are the mesenchymal stromal cells of any one of claims 41-50.

60. The method of any one of claims 53-59, wherein the mesenchymal stromal cells are administered in combination with an allogeneic transplanted cell or tissue.

61. The method of claim 60, wherein the cell a retinal pigment epithelium cell, retinal cell, corneal cell, or muscle cell.

Abstract

The present disclosure generally relates to methods of generating mesenchymal stromal cells from hemangioblasts, e.g., hemangioblasts differentiated from ESCs. These methods produce substantial numbers of high quality mesenchymal stromal cells. The resulting mesenchymal stromal cells are useful in the treatment various diseases and conditions such as multiple sclerosis and other autoimmune disorders.

Figure 1: Generation of MSCs from hESC via hemangioblasts

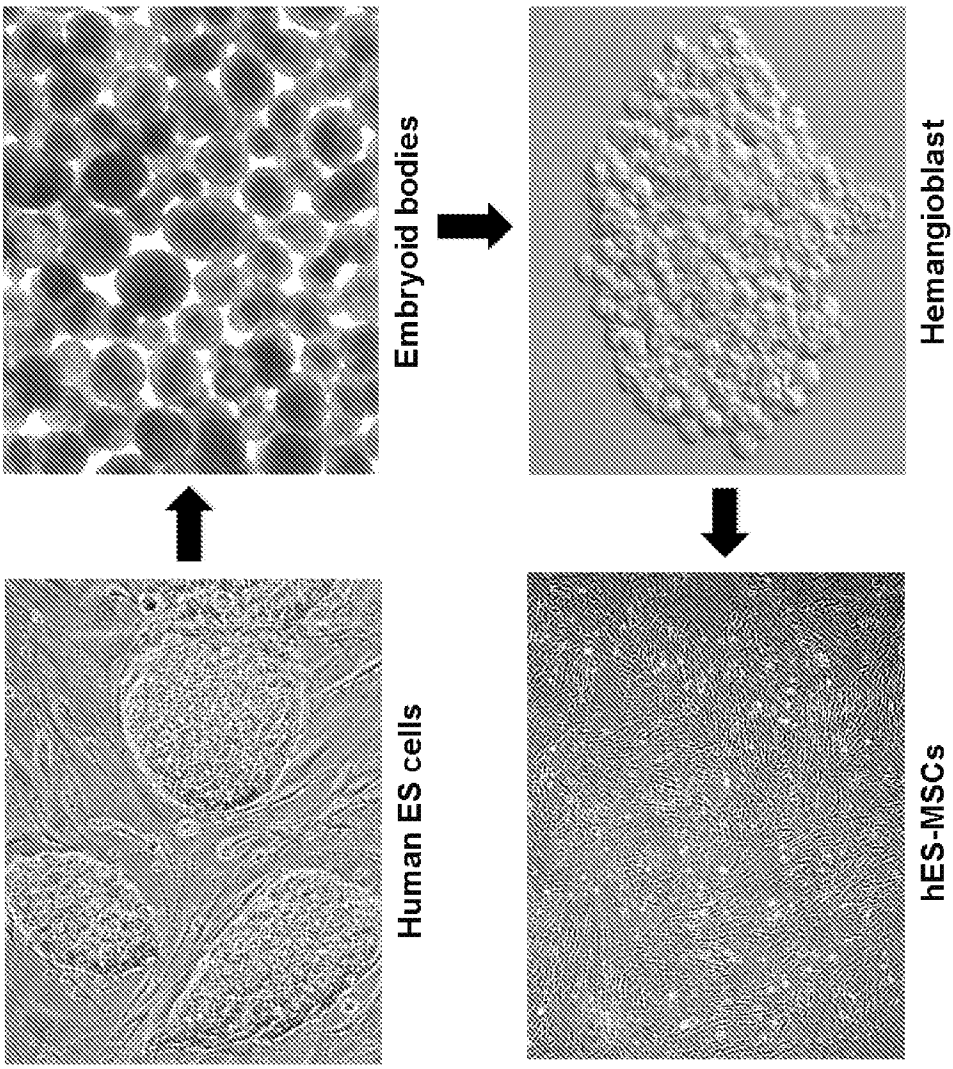
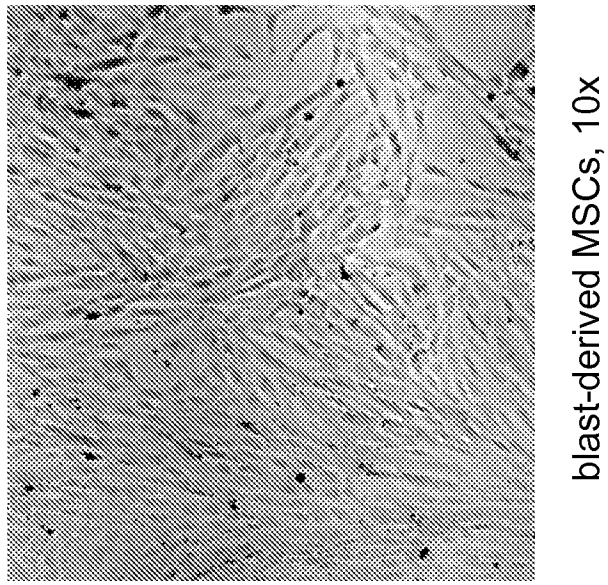


Figure 2: MSCs obtained from hESC-derived hemangioblasts



blast-derived MSCs, 10x

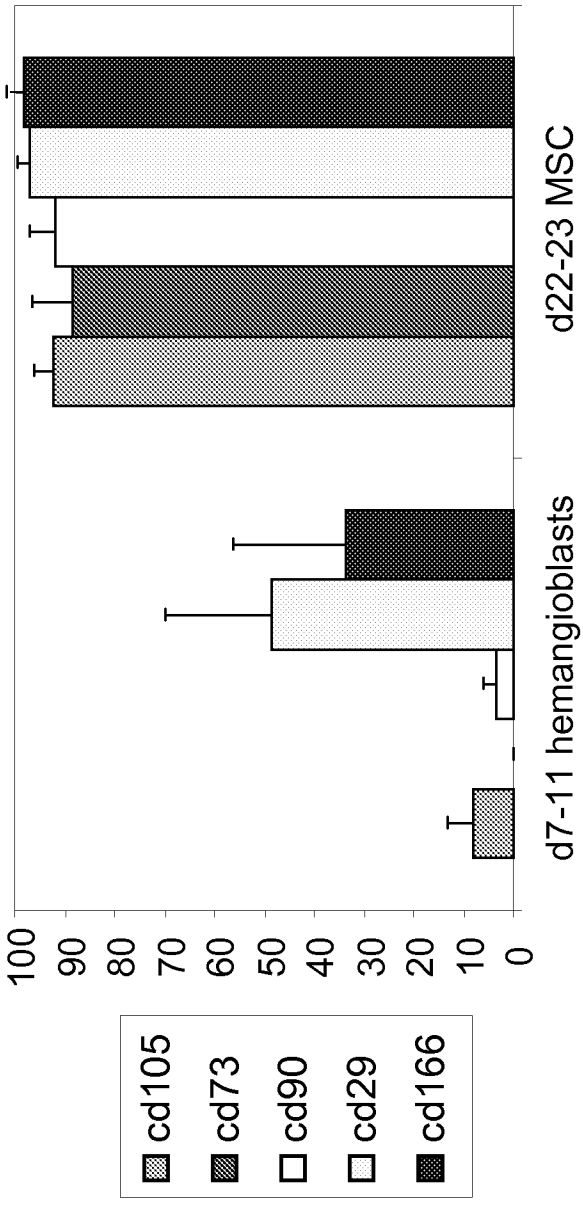


Figure 3: Immunophenotype of MSCs derived from different culture methods

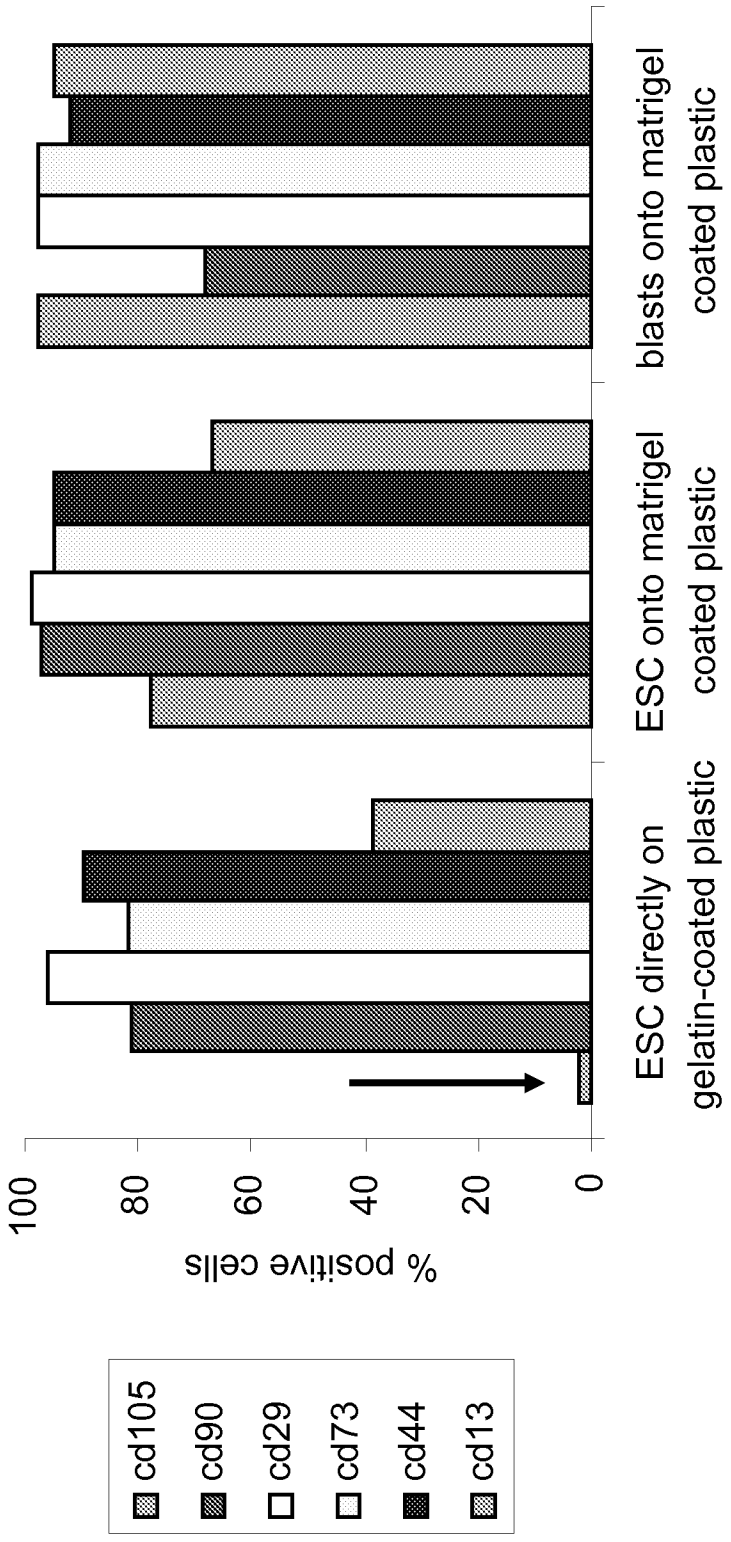


Figure 4: MSC yield from hESCs varies based on derivation method

	ESC directly on gelatin-coated plastic	ESC onto matrigel-coated plastic	blasts onto matrigel-coated plastic
# of starting hESCs:	300,000	350,000	~200,000
yield :	n/a	4 million	85 million
yield collected at:	n/a	48 days	44 days

Figure 5: Acquisition of MSC surface markers is faster using hemangioblast method than it is for direct hESC to MSC method

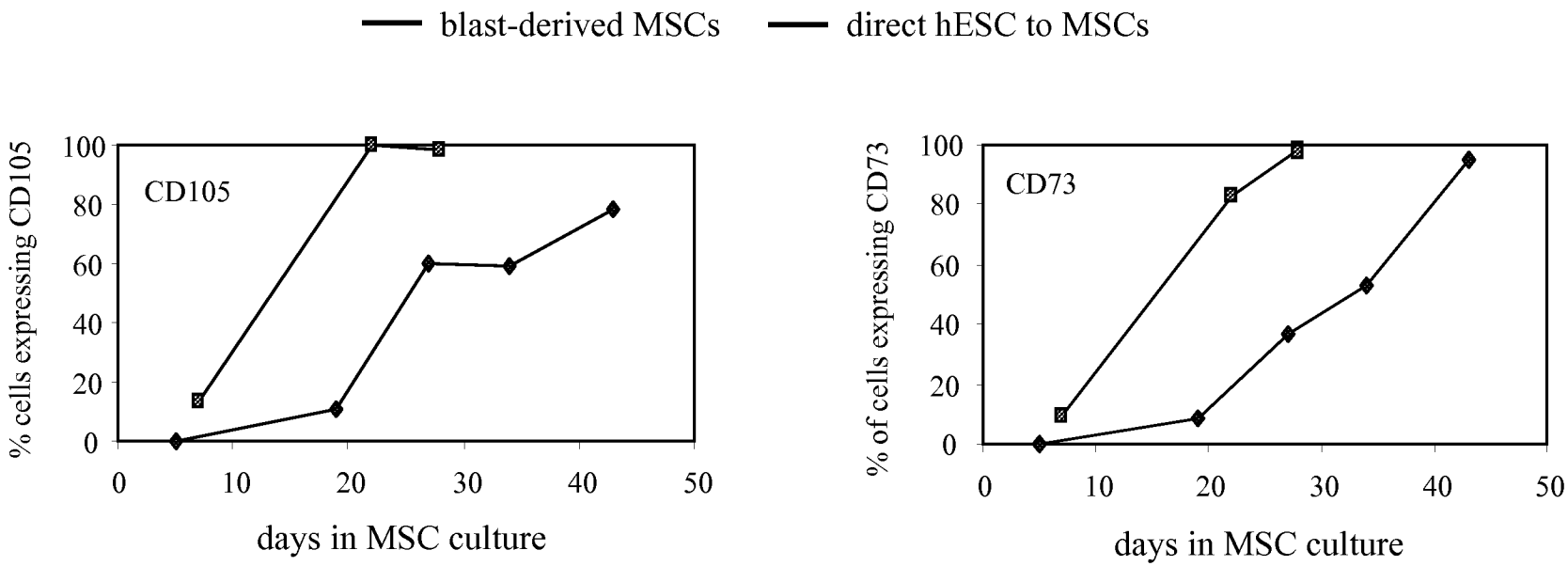


Figure 6: MSCs are HLA-ABC$^+$ and CD34$^-$, CD45$^-$

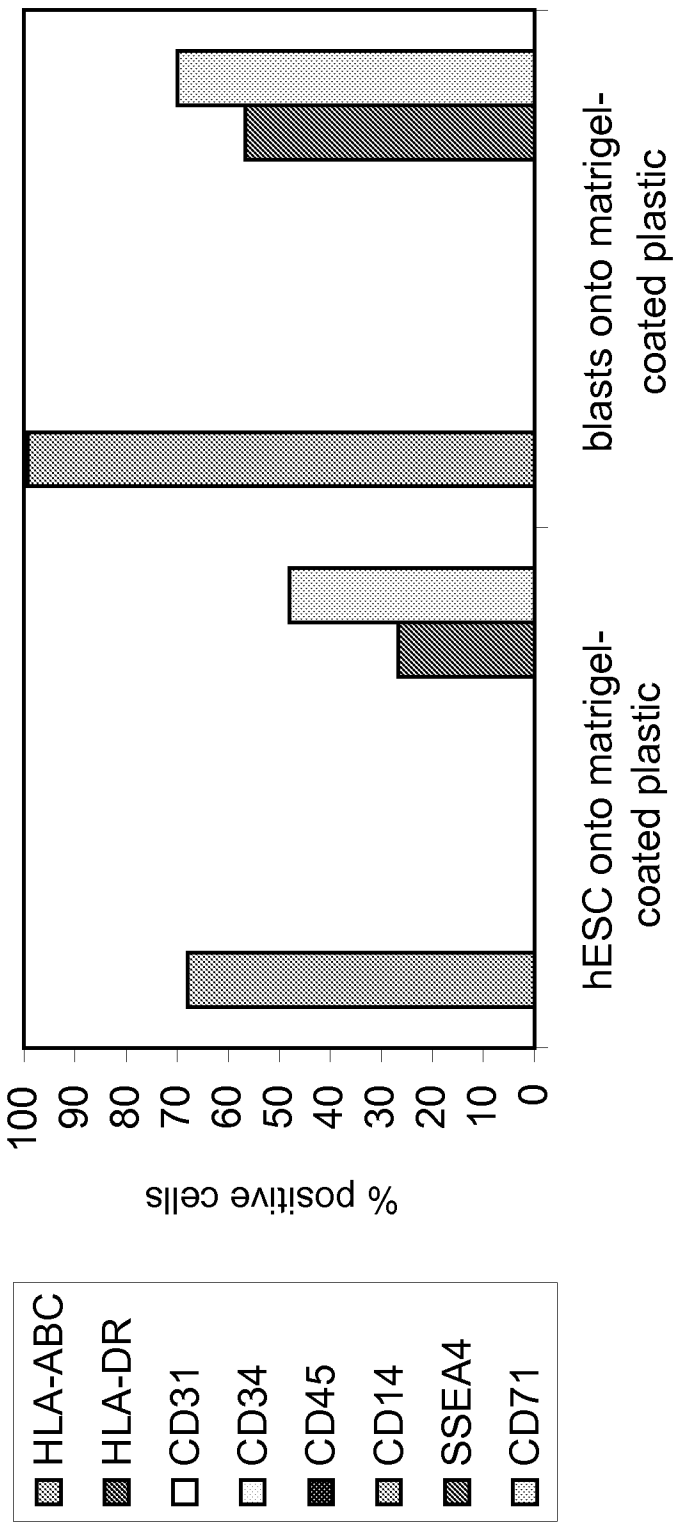
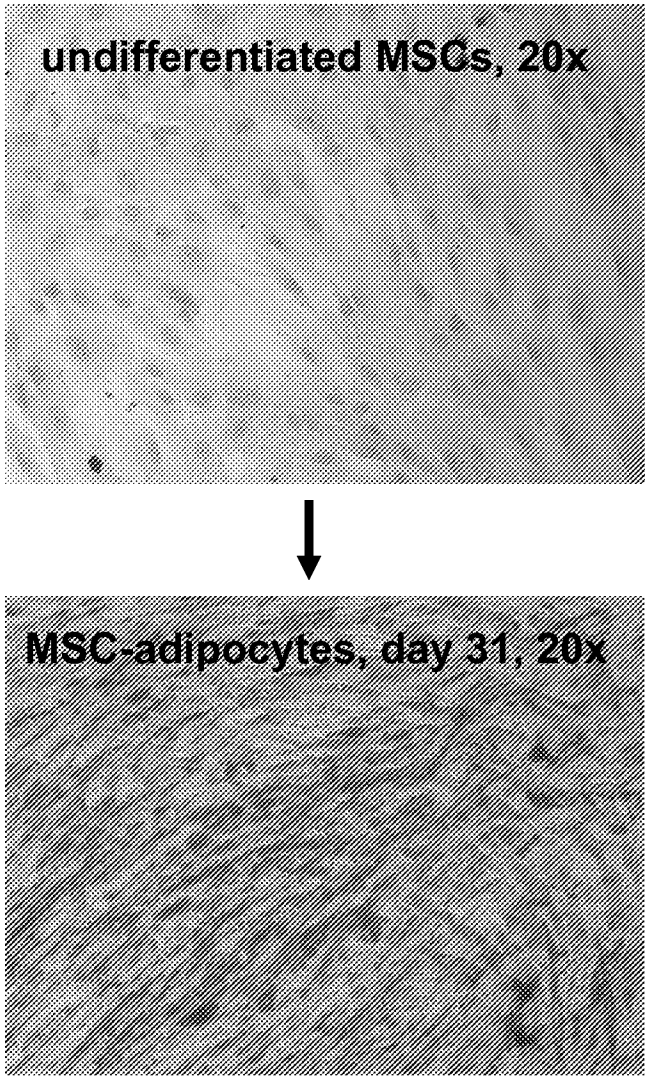


Figure 7:
hESC-derived MSCs display differentiation capabilities

Oil Red O for adipocytes



Alizarin Red for osteocytes

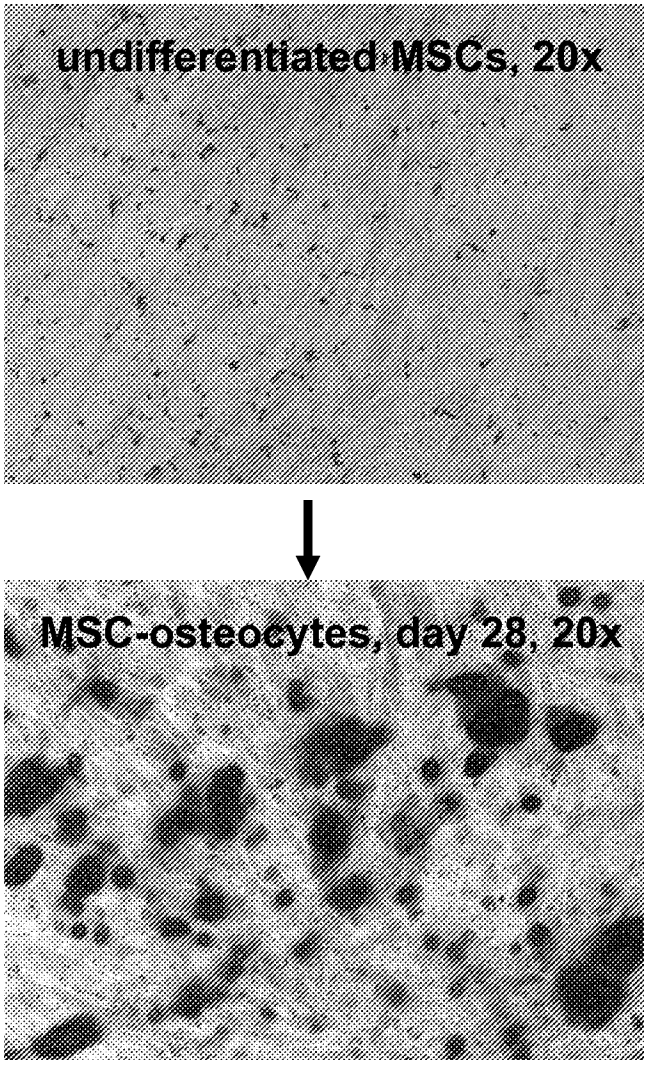
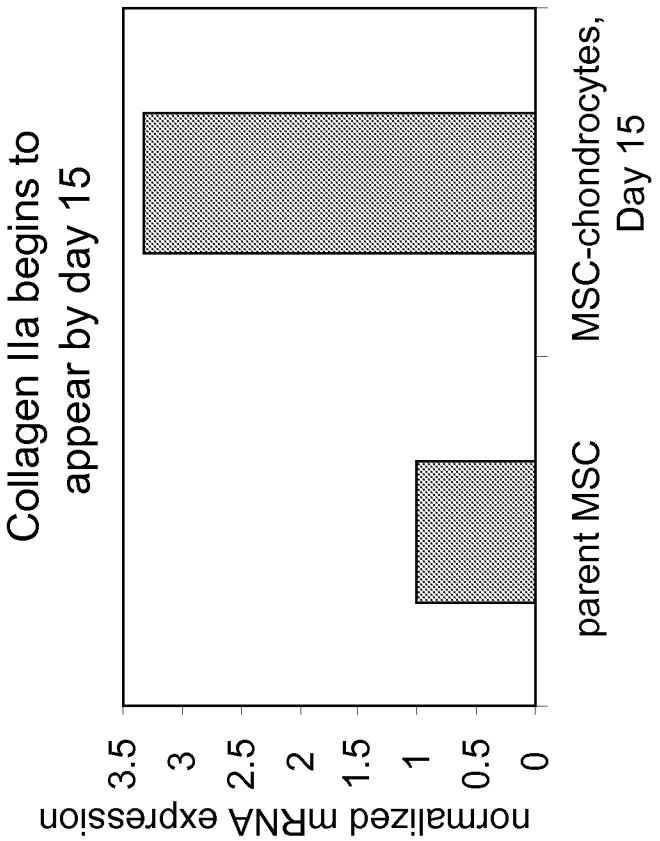


Figure 8: MSC chondrogenic differentiation



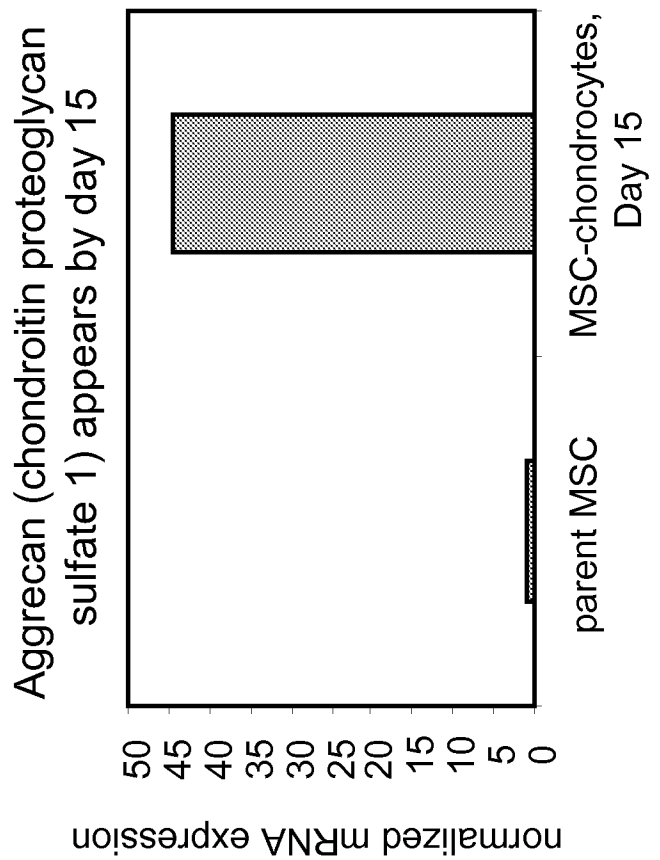
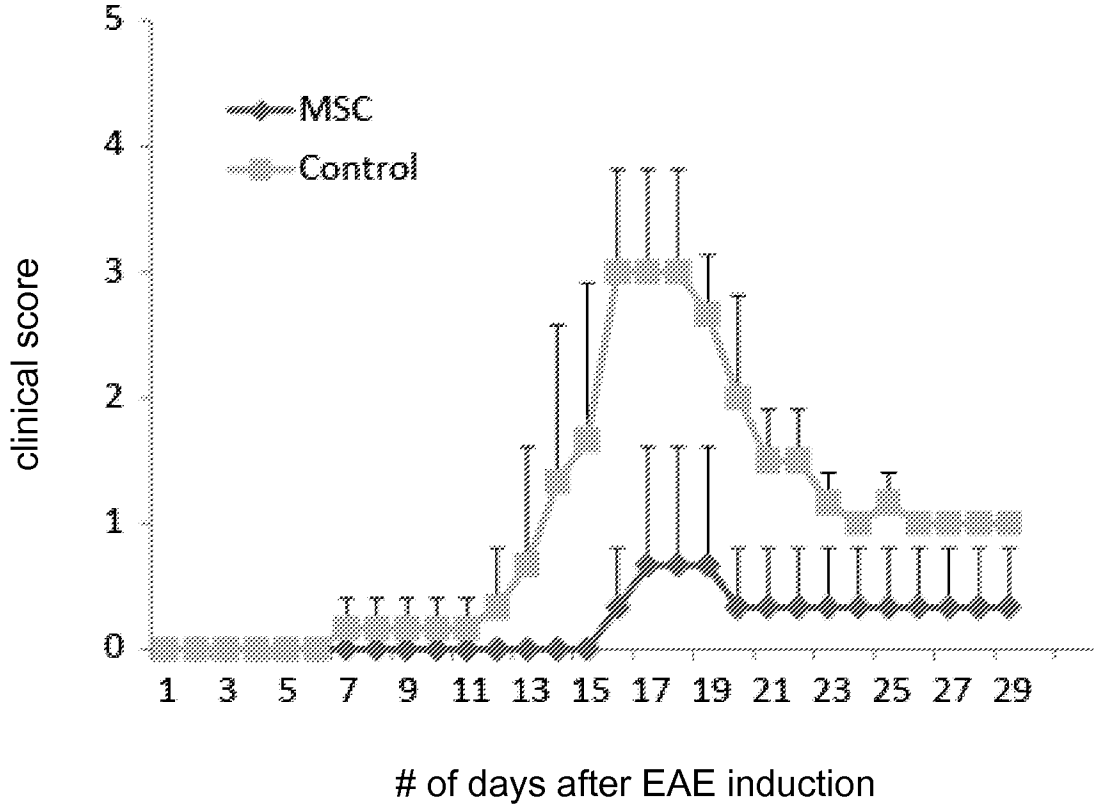


Figure 9: One injection of hESC-MSCs can reduce the score of multiple sclerosis symptoms in EAE mouse model



Experimental autoimmune encephalomyelitis (EAE) is delayed in mice treated with hESC-MSCs (n = 3), versus control mice treated with vehicle (n = 4). Clinical score was recorded daily following EAE induction by immunization of the mice with the MOG antigen.

Figure 10: Transient expression of CD309 may be an indicator of MSC precursor state

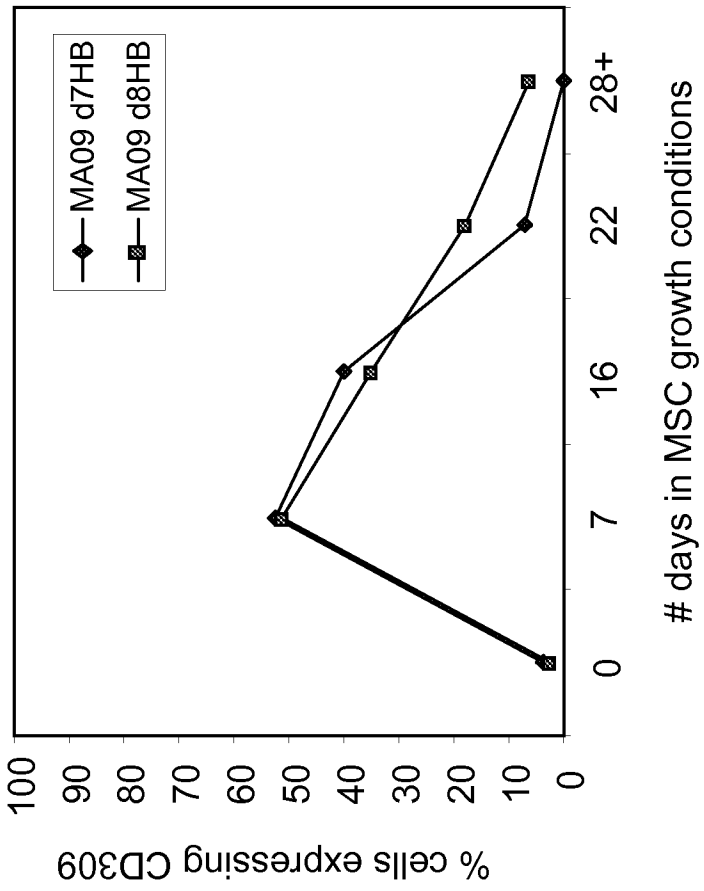


Figure11a: T cell proliferation in response to mitogenic stimulus is suppressed by hESC-MSCs

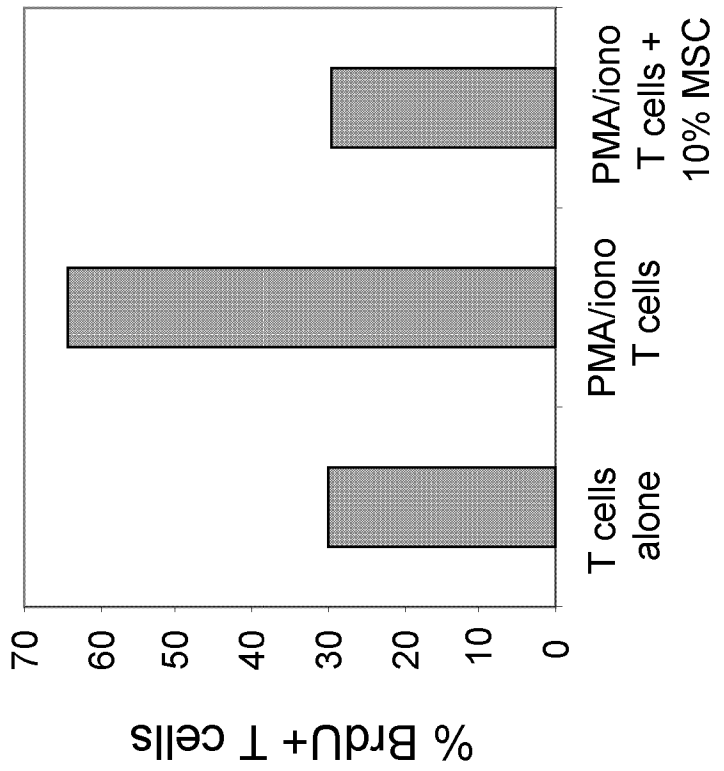
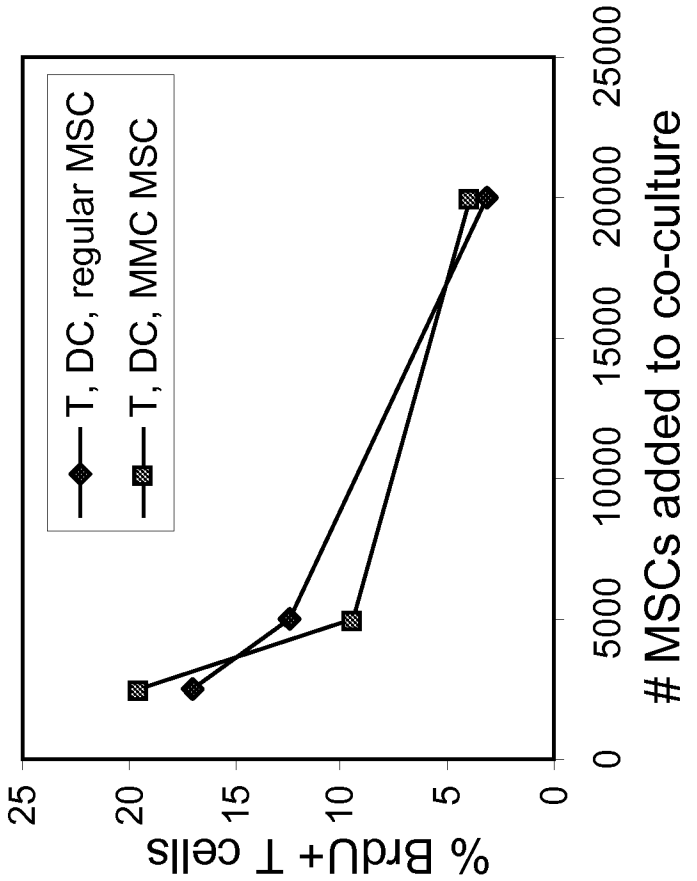


Figure 11b: T cell proliferation in response to antigen presenting cells (DCs) is suppressed by hESC-MSCs



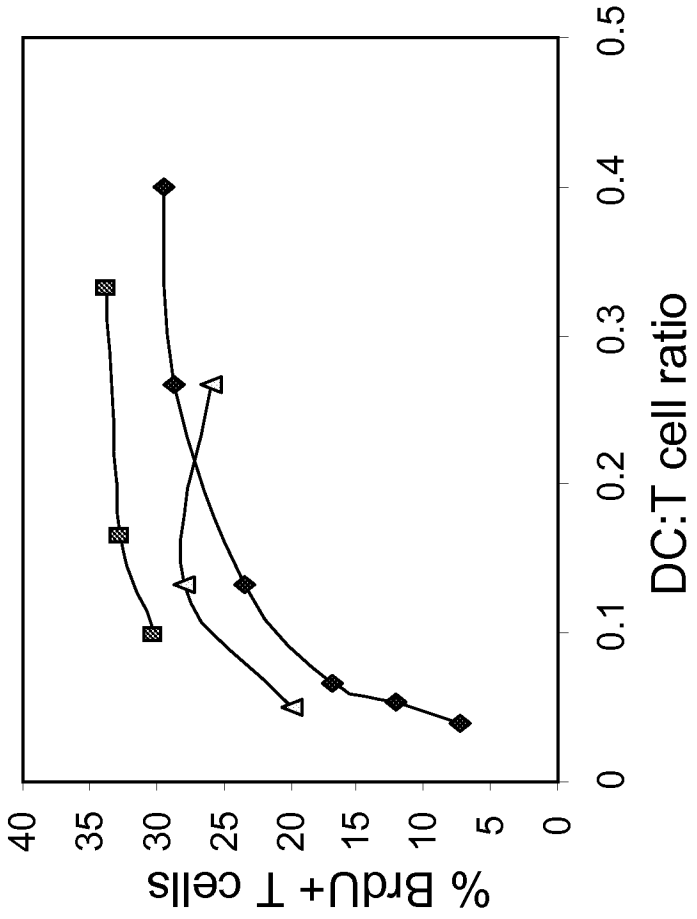
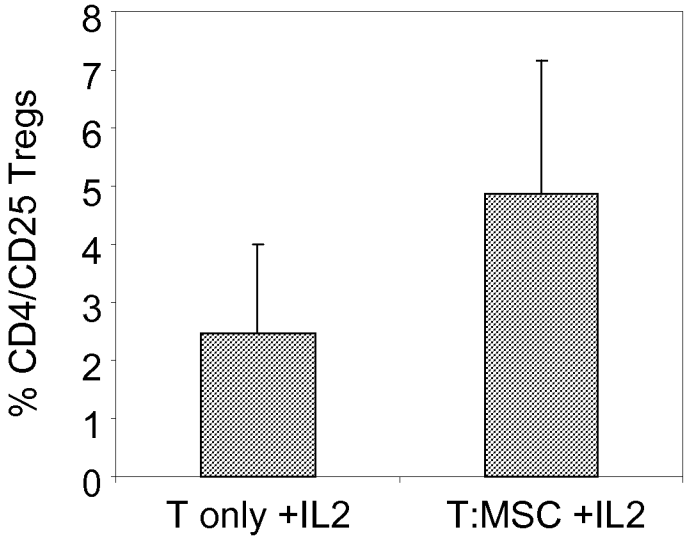


Figure 12: hESC-MSCs influence T cell subsets

A. hESC-MSCs increase Treg population



B. hESC-MSCs inhibit Th1 secretion of IFNγ

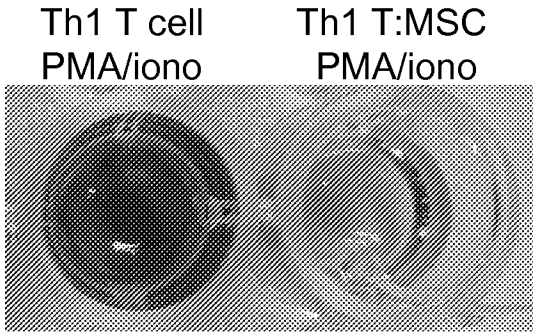


Figure 13: The pro-inflammatory cytokine, interferon gamma, stimulates changes in MSC surface marker expression and may enhance MSC immunosuppressive effects

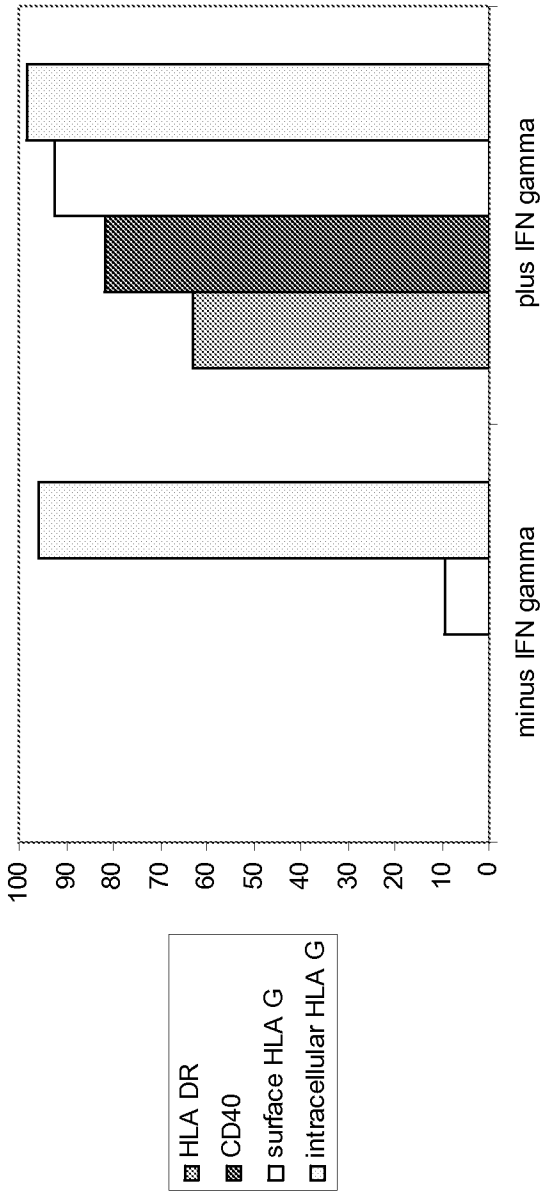


Figure 1: Generation of MSCs from hESC via hemangioblasts

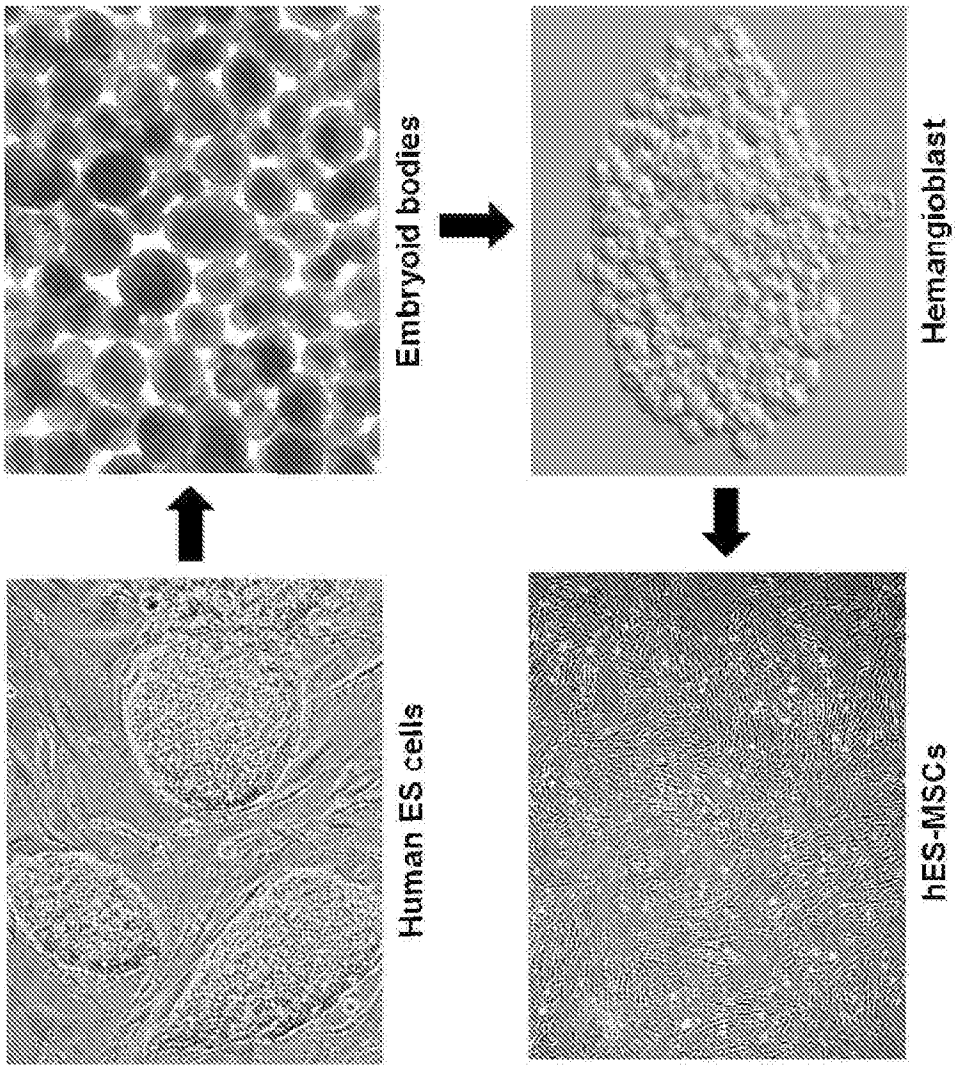
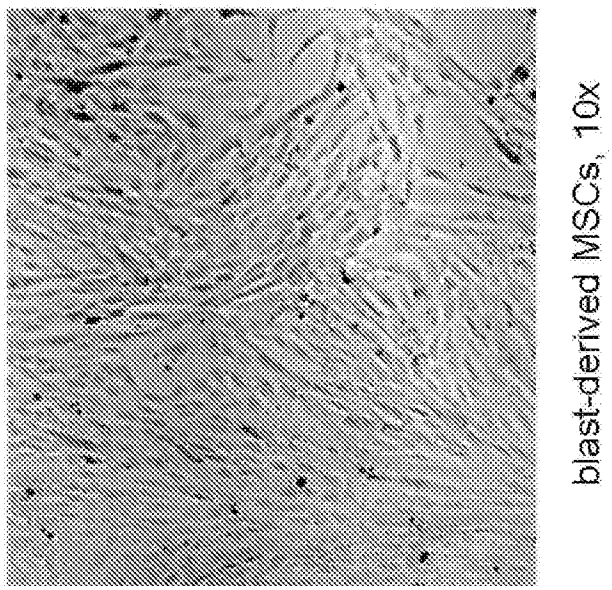


Figure 2: MSCs obtained from hESC-derived hemangioblasts



blast-derived MSCs, 10x

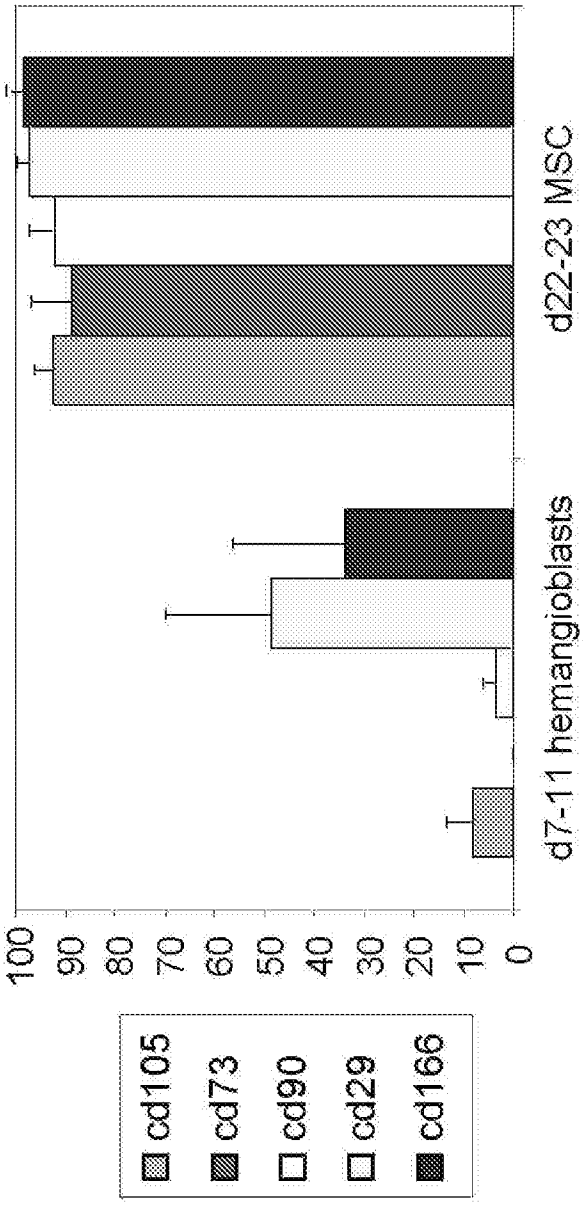


Figure 3: Immunophenotype of MSCs derived from different culture methods

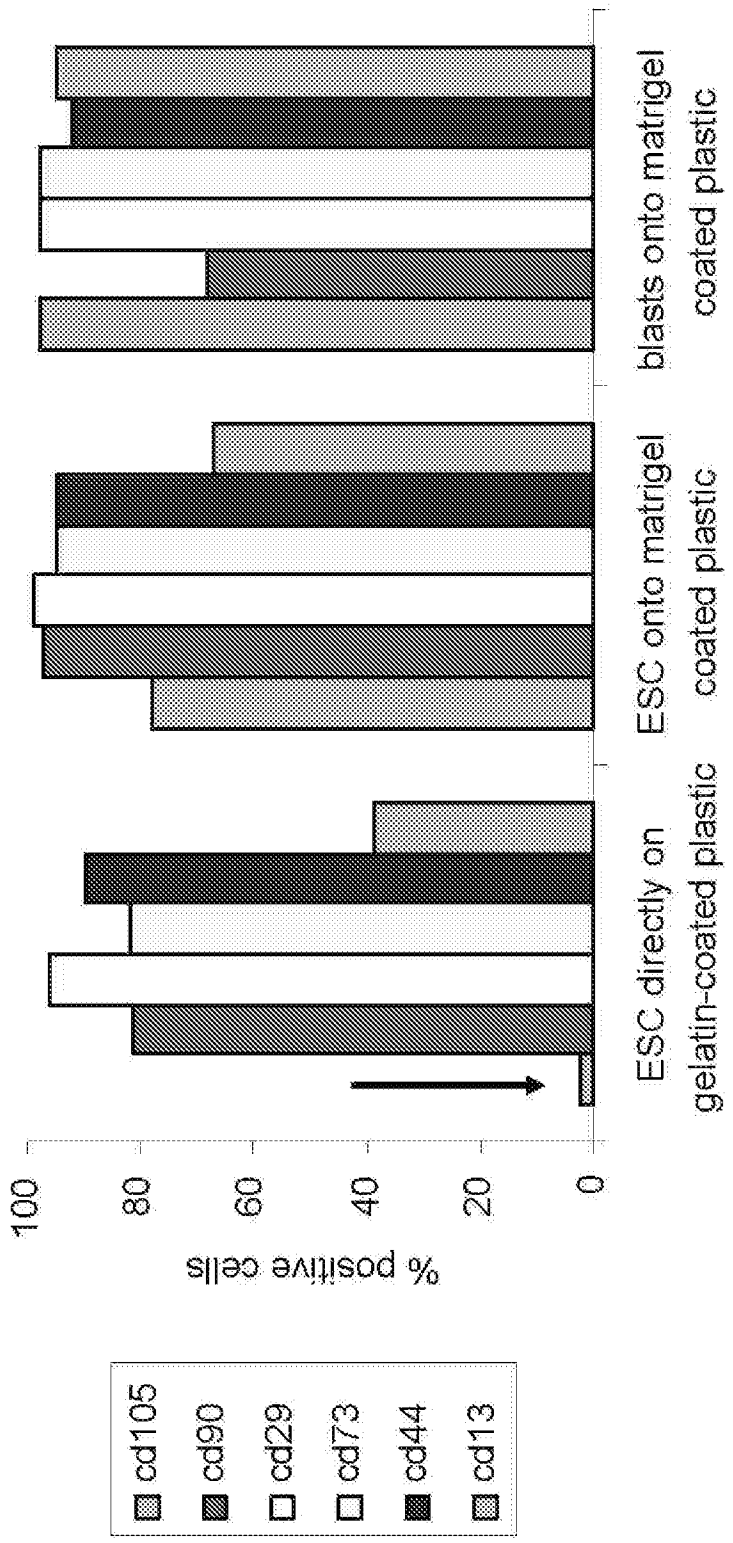


Figure 4: MSC yield from hESCs varies based on derivation method

	ESC directly on gelatin-coated plastic	ESC onto matrigel-coated plastic	blasts onto matrigel-coated plastic
# of starting hESCs:	300,000	350,000	~200,000
yield :	n/a	4 million	85 million
yield collected at:	n/a	48 days	44 days

Figure 5: Acquisition of MSC surface markers is faster using hemangioblast method than it is for direct hESC to MSC method

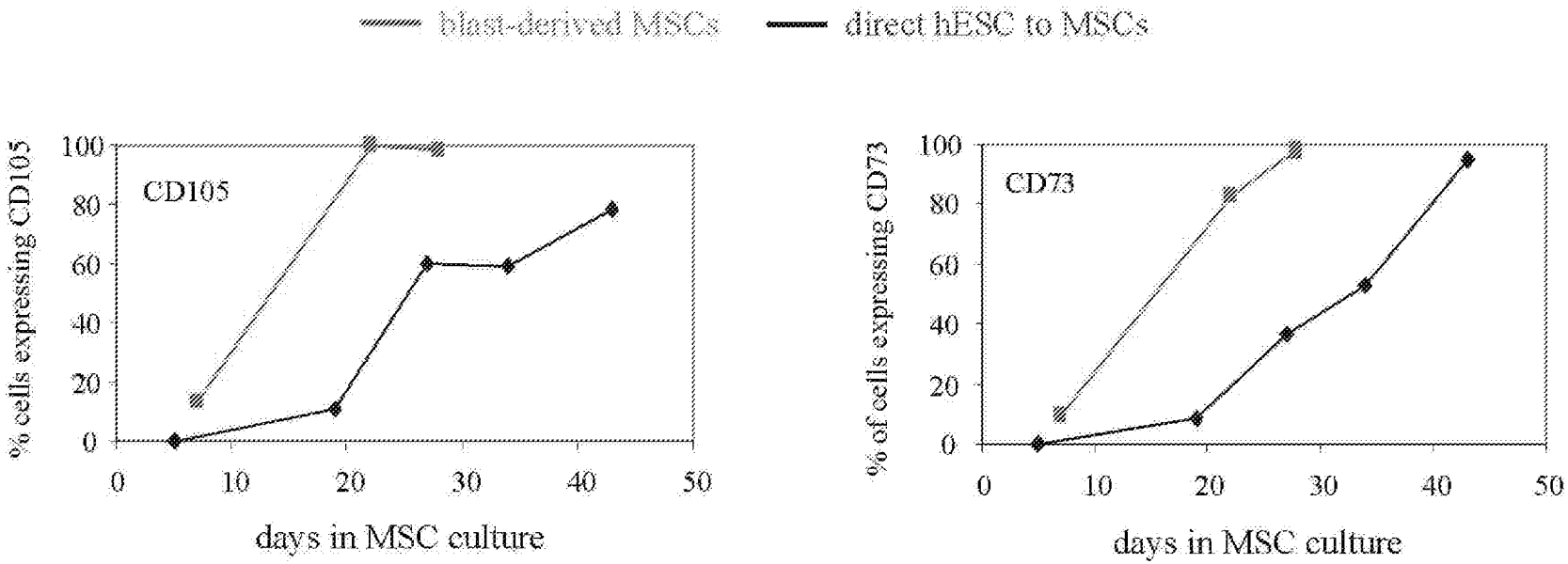


Figure 6: MSCs are HLA-ABC$^+$ and CD34$^-$, CD45$^-$

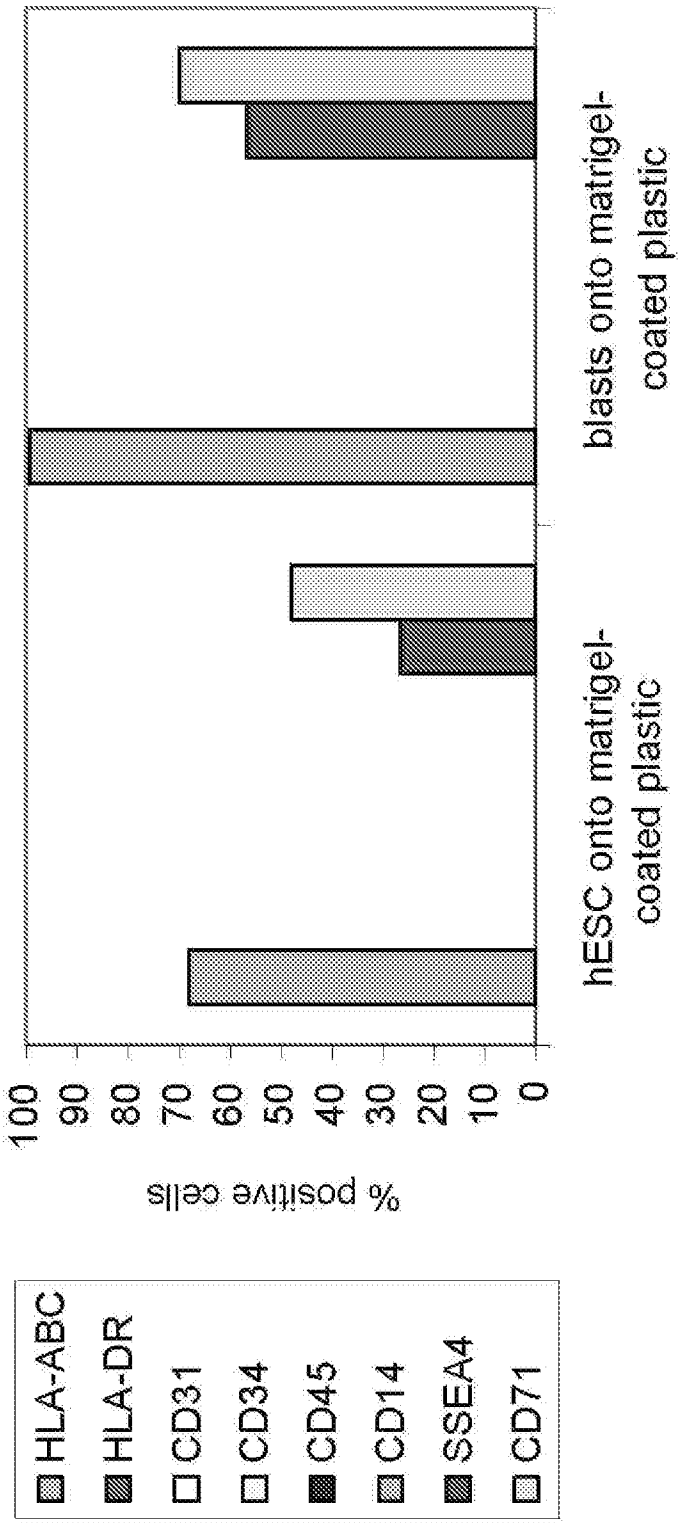


Figure 7:
hESC-derived MSCs display differentiation capabilities

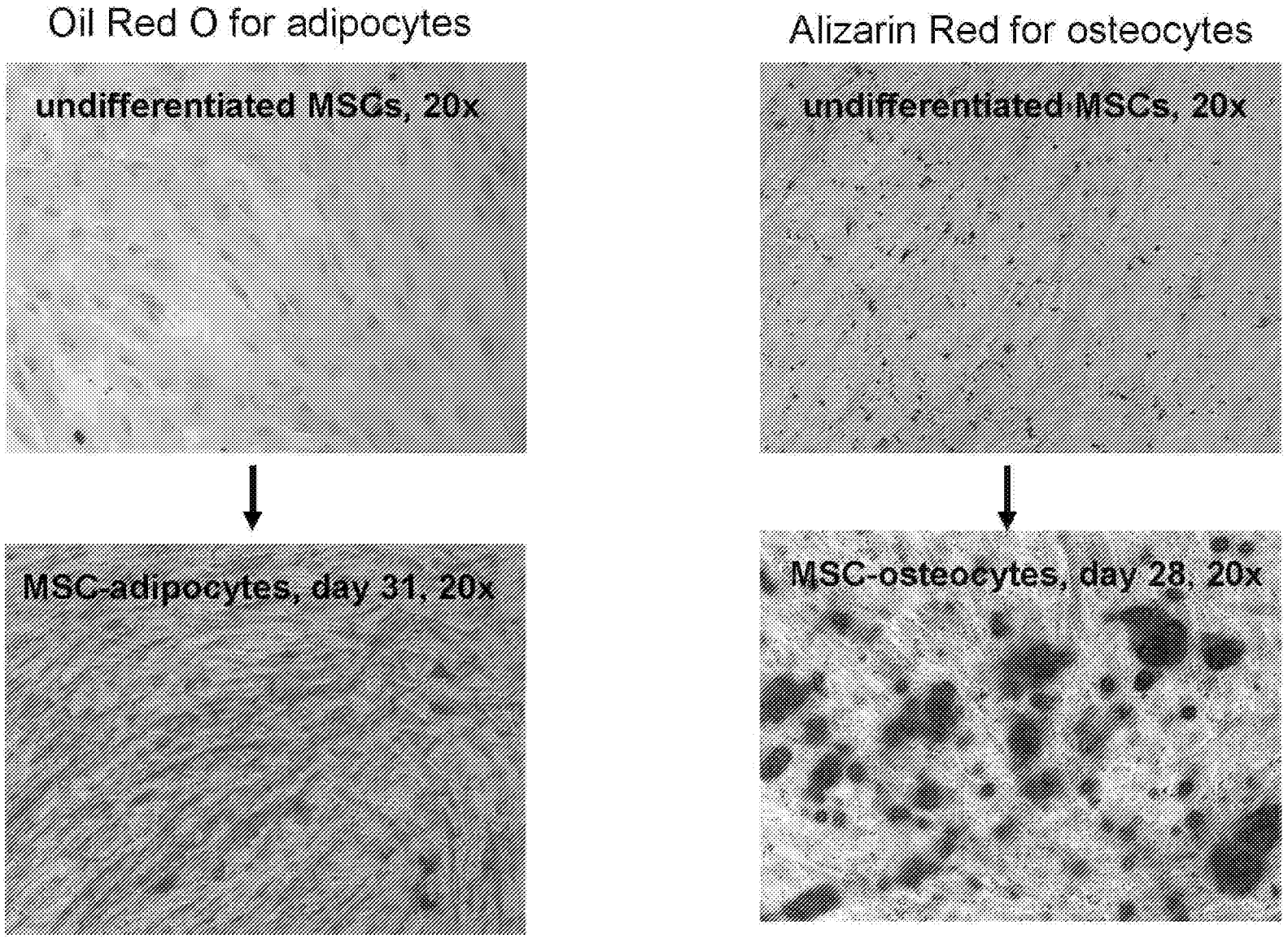
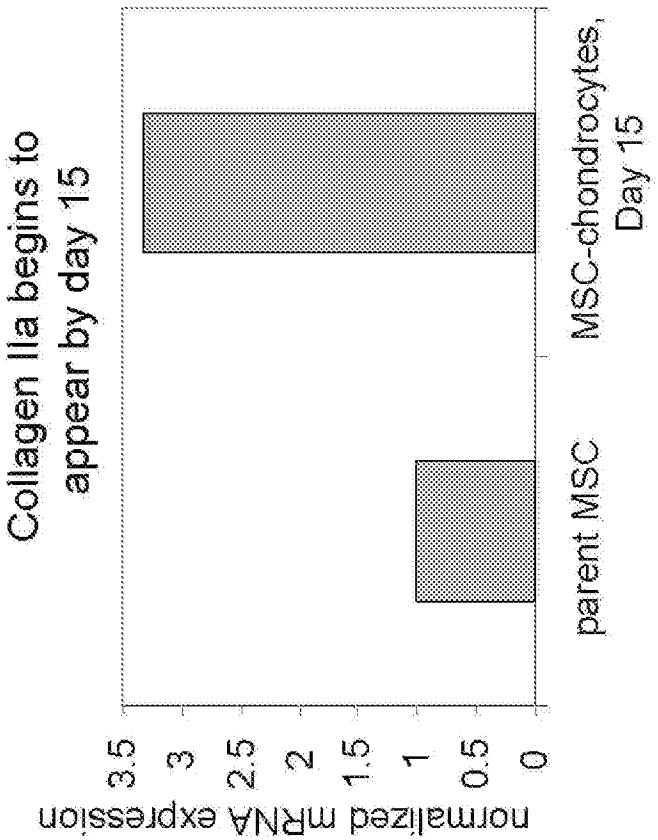


Figure 8: MSC chondrogenic differentiation



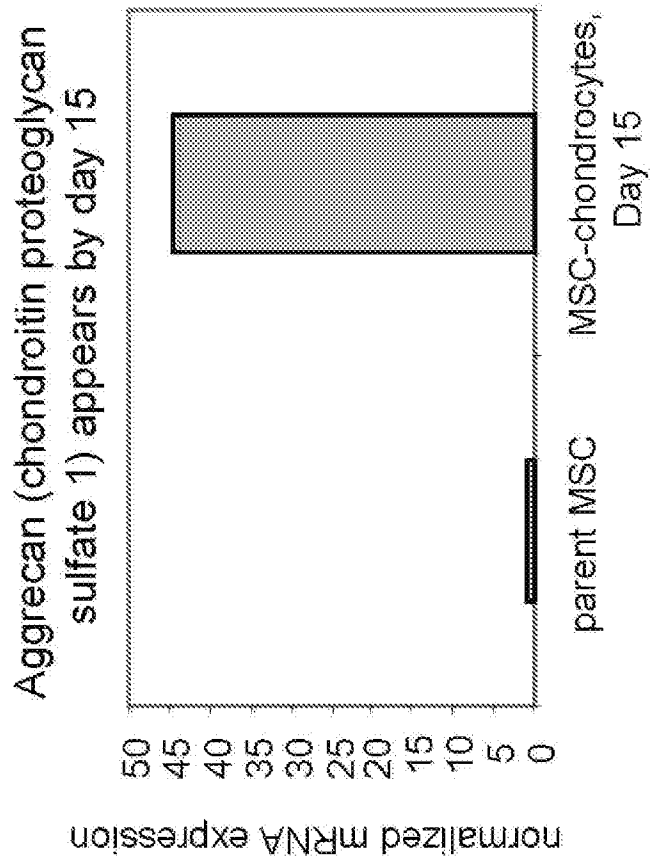
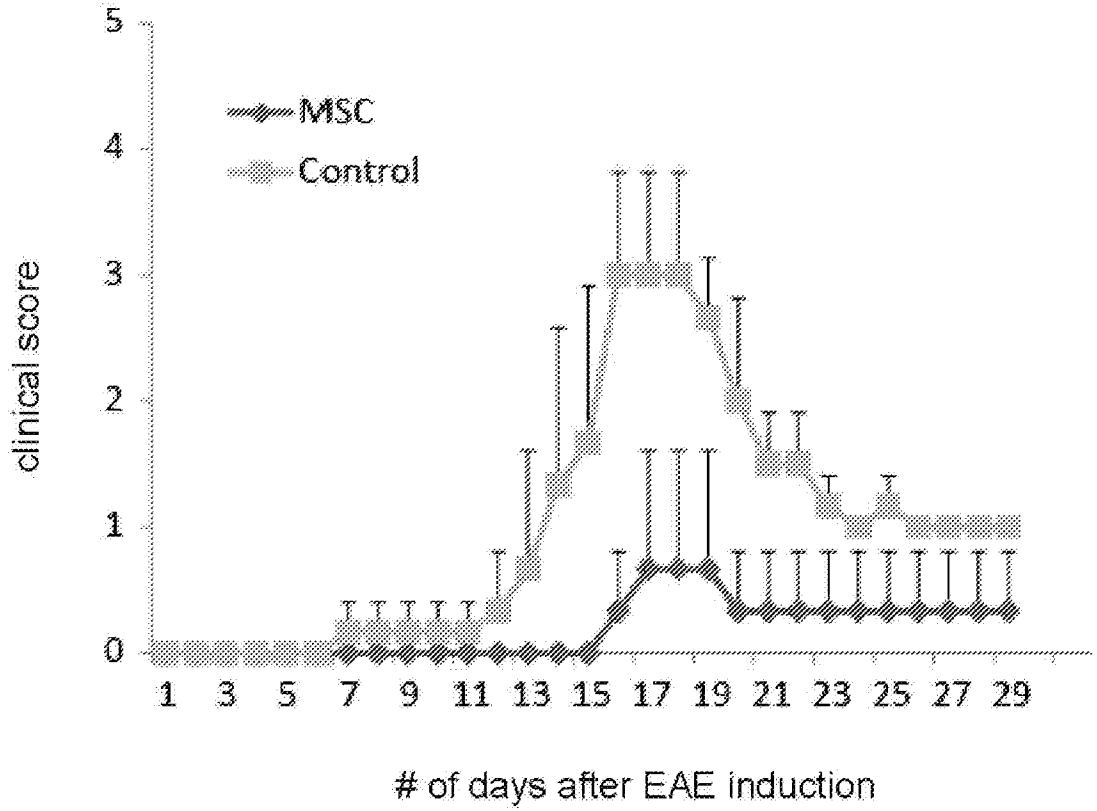


Figure 9: One injection of hESC-MSCs can reduce the score of multiple sclerosis symptoms in EAE mouse model



Experimental autoimmune encephalomyelitis (EAE) is delayed in mice treated with hESC-MSCs (n = 3), versus control mice treated with vehicle (n = 4). Clinical score was recorded daily following EAE induction by immunization of the mice with the MOG antigen.

Figure 10: Transient expression of CD309 may be an indicator of MSC precursor state

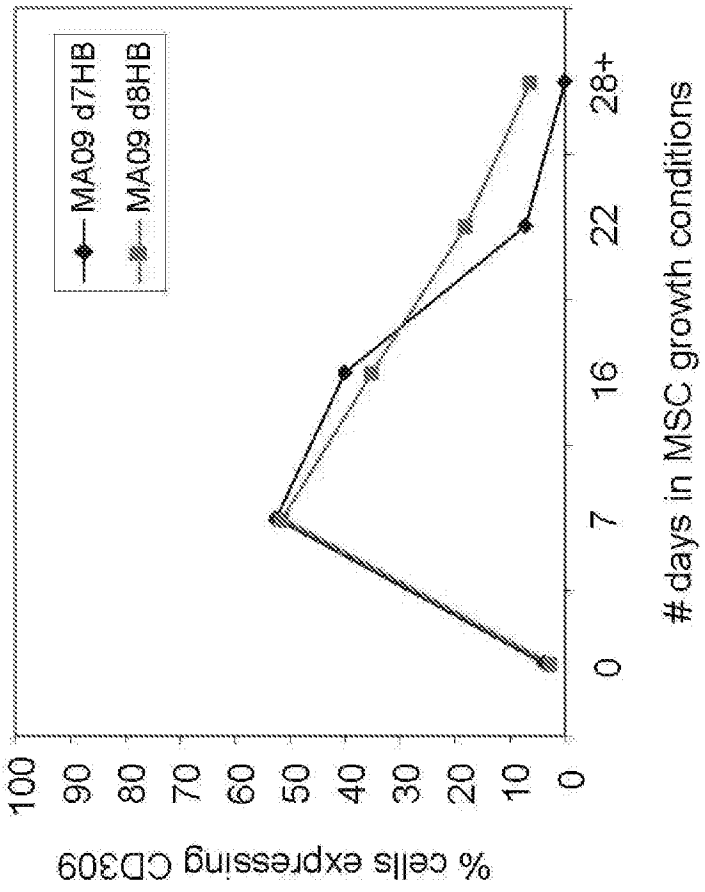


Figure11a: T cell proliferation in response to mitogenic stimulus is suppressed by hESC-MSCs

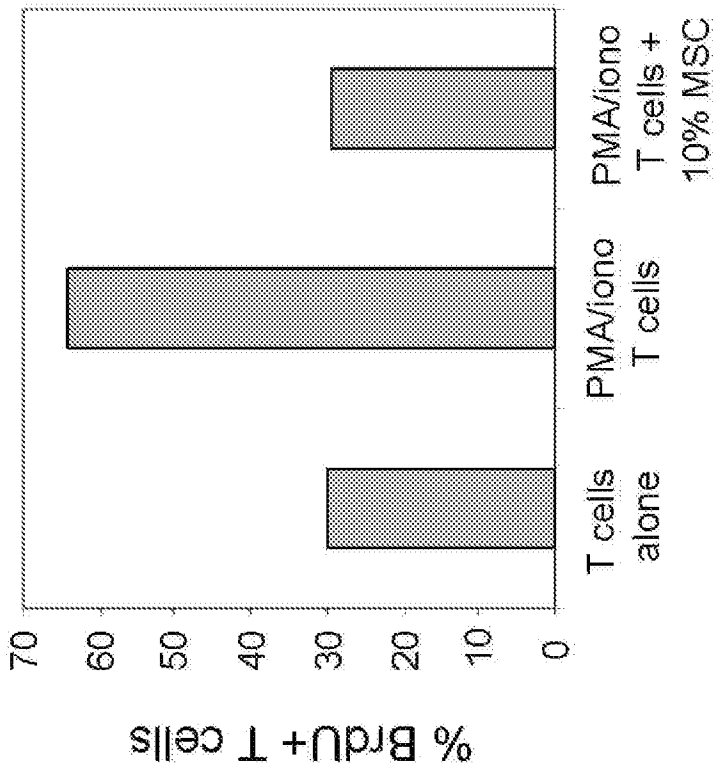


Figure 11b: T cell proliferation in response to antigen presenting cells (DCs) is suppressed by hESC-MSCs

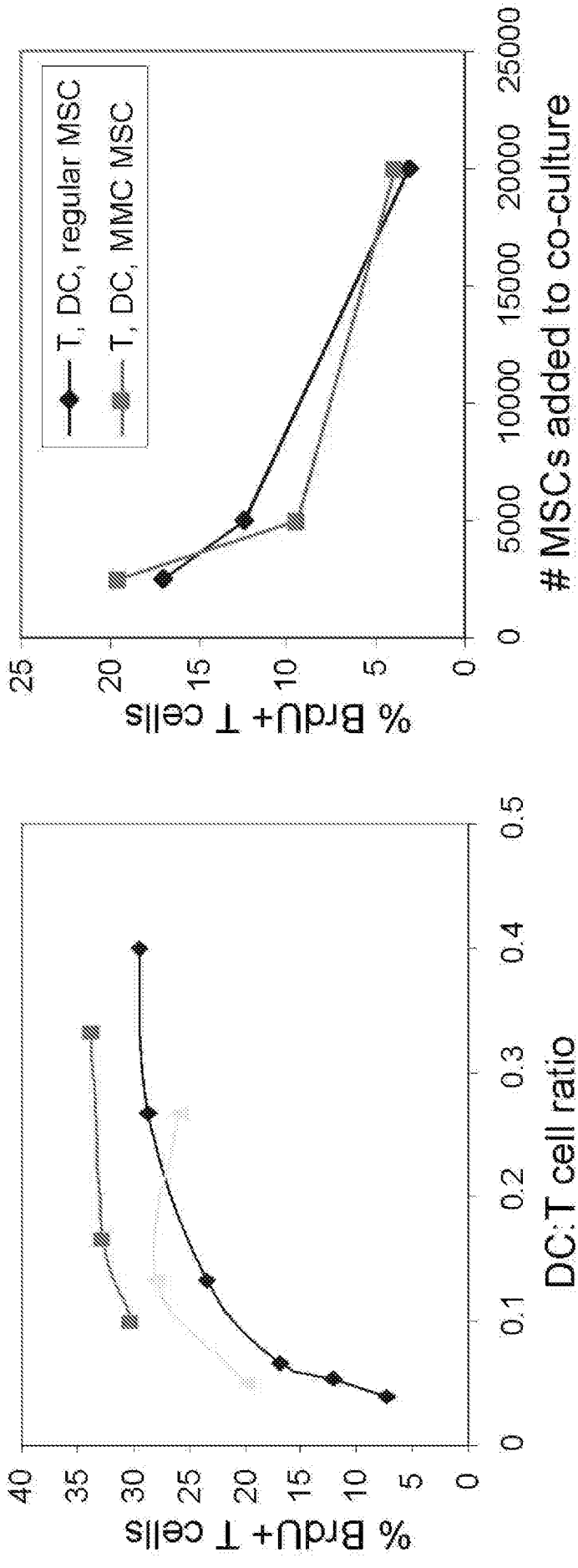
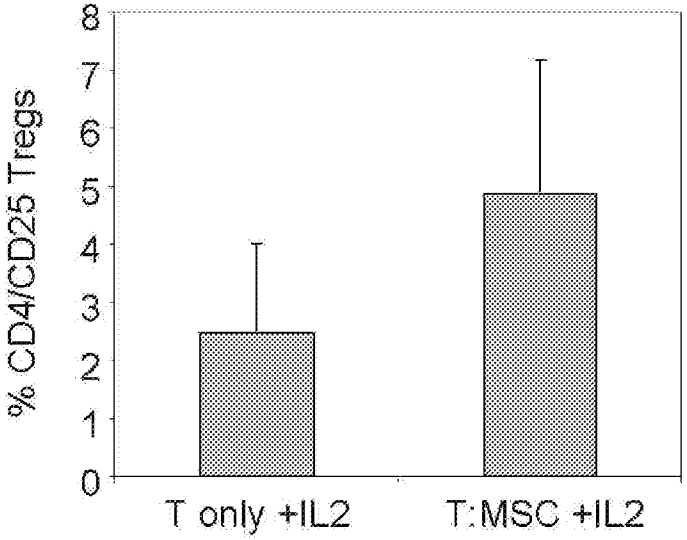


Figure 12: hESC-MSCs influence T cell subsets

A. hESC-MSCs increase Treg population



B. hESC-MSCs inhibit Th1 secretion of IFNγ

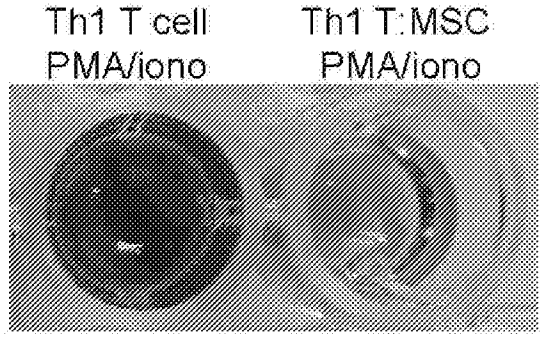
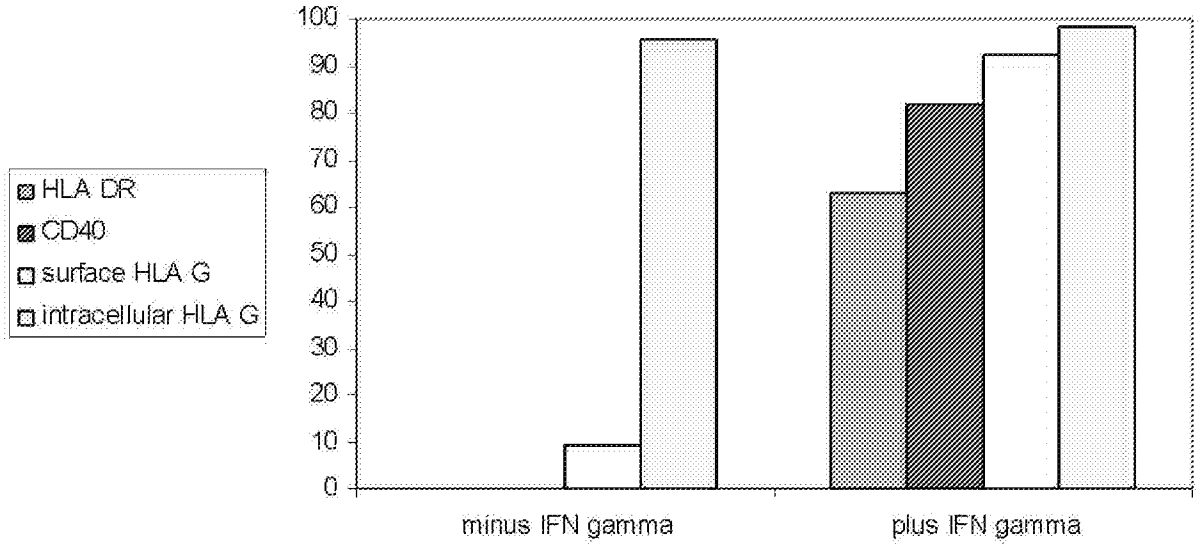


Figure 13: The pro-inflammatory cytokine, interferon gamma, stimulates changes in MSC surface marker expression and may enhance MSC immunosuppressive effects



Electronic Acknowledgement Receipt

EFS ID:	11515829
Application Number:	61565358
International Application Number:	
Confirmation Number:	1601
Title of Invention:	METHODS OF GENERATING MESENCHYMAL STROMAL CELLS USING HEMANGIOBLASTS
First Named Inventor/Applicant Name:	Erin A. Kimbrel
Customer Number:	21967
Filer:	Alexander Harrison Spiegler/Gwen Peacher
Filer Authorized By:	Alexander Harrison Spiegler
Attorney Docket Number:	75820.210001
Receipt Date:	30-NOV-2011
Filing Date:	
Time Stamp:	18:38:03
Application Type:	Provisional

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$125
RAM confirmation Number	7502
Deposit Account	500206
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Transmittal of New Application	75820ProvTransmittal.pdf	56570 946851e936b186bd45e20e4aeeefa5caae44e8df	no	1
Warnings:					
Information:					
2	Specification	specification.pdf	2149570 a23a1e0fab541ca3dd135e5c8761bb01bbe15245	no	51
Warnings:					
Information:					
3	Drawings-only black and white line drawings	BWdrawings.pdf	2217513 3bf5b97f695b3aab2530c849b2e9749adc93e6d6	no	14
Warnings:					
Information:					
4	Drawings-other than black and white line drawings	drawingscolor.pdf	2519390 50f204295260d00ef61060aa23a8da70c032a07e	no	14
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	29889 966c9e6e8a2042589f8b969ecb1d16889f45742f	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	6972932		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

DocCode - SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 61565358 Document Date: 11/30/2011

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

- Drawings

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

To access the documents in the SCORE database, refer to instructions developed by SIRA.

At the time of document entry (noted above):

- Examiners may access SCORE content via the eDAN interface using the Supplemental Content tab.
- Other USPTO employees can bookmark the current SCORE URL (http://es/ScoreAccessWeb/).
- External customers may access SCORE content via the Public and Private PAIR interfaces using the Supplemental Content tab.

Form Revision Date: May 1, 2009